Cellular Immune Responses Of Marsupials: 
Family Macropodidae

Lauren Jill Young
B. Sc. (Hons)
Grad. Dip. Ed.

A thesis submitted to the University of Western Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy

School of Science, Food and Horticulture
University of Western Sydney
2002
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
"Sadly, our knowledge of the biology of many threatened mammals is so slight that we do not yet have the information necessary for rational programs of management"

A. A. Burbidge in 'Mammals of Australia'
Summary

This thesis describes a comprehensive study of the cellular responses of a number of endangered marsupial species with a principal focus on the tammar wallaby (Macropus eugenii) as a model macropod species. The development of in vitro experimental assays for the assessment of immune responses in this model species are described, which provided a set of benchmarks for comparisons with other members of the Macropodidae and with eutherian mammals. Once this data was collected and protocols were established, the study was extended to include investigations of the immune responses in opportunistic samples obtained from the Rufous Hare-wallaby (Lagorchestes hirsutus), the Long-footed potoroo (Potorous longipes) and the more common, but nonetheless still vulnerable, Long-nosed potoroo (Potorous tridactylus) with a view to investigating their apparent susceptibility to infection with intracellular pathogens, particularly mycobacterial species.

The overall findings from the application of these assays suggest that the cellular immune responses of these species are relatively complex and involve a level of sophistication that rivals their eutherian counterparts. Specifically, peripheral blood and tissue leukocytes were morphologically similar to those of other mammals, with the exception of tammar wallaby monocytes that appeared to contain few lysosomal granules, and the basophils of the Rufous Hare-wallaby that contained very large atypical granules.

Monocytes and granulocytes isolated from these species demonstrated the capacity to respond, migrate toward, ingest and destroy foreign material, with the exception of captive-born potoroos that failed to generate a response to endotoxin in these tests.

Lymphocyte proliferation responses were documented using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colourimetric assay, a proliferation assay used in place of the tritiated thymidine technique and applied for the first time to the measurement of marsupial cell proliferation. Results from this study demonstrated that marsupial peripheral blood mononuclear cells (PBMC) are able to grow and proliferate in biochemically supplemented serum-free media when
treated with the T cell mitogens, PHA and PWM, without the large standard deviations and variations previously reported in serum-supplemented media. Immunoregulatory factors secreted into the supernatants of these cultures displayed both stimulatory and inhibitory activity and were further characterised at the molecular level using RT-PCR. The expression of the cytokines TNF-α, IL-1β and IL-10 was detected in the mitogen-activated PBMC of the tammar wallaby and partial sequence information was also obtained for cDNA prepared from the peripheral blood and tissues of the other macropod species studied in this work. TNF-α, IL-10 and Type I IFNs were amplified from the PBMC and spleen and lung tissues of the Long-footed potoroo, TNF-α and IL-10 were amplified from the PBMC of the Long-nosed potoroo and TNF-α was amplified from the PBMCs of the Rufous Hare-wallaby. IL-1β was not detected in these tissues.

Histological and immunohistological investigations using standard haematoxylin and eosin techniques and species cross-reactive antibodies revealed that the lymphoid tissues of these three species were similar to those of eutherian mammals and to other macropod species described thus far. Acid-fast staining revealed localisation of mycobacteria to phagocytes within these tissues, although these organisms were also detected in the liver and kidney of one animal and were scattered throughout the tissues of the most severely affected hosts. Immunopathology of animal tissues included granuloma formation in the skin, spleen, lungs, adrenal gland and liver of some animals and the presence of multinucleated giant cells in the lungs of others.

The overall findings of this study suggest that the immune systems of macropod species possess most of the sophistication associated with that of eutherian mammals. Whilst some differences were apparent in cells and their products in the test species, no single factor common to all macropods was identified as a cause for immune dysfunction. It appears likely that as yet undefined factors related to their confinement rather than an inherent defect in their immunocapacity is responsible for the apparent disease susceptibility of these animals.
Acknowledgements

I would like to thank Professor Elizabeth Deane and the University of Western Sydney for the opportunity to study marsupial immunology. I gratefully acknowledge the University of Western Sydney and the Department of Education and Training for an Australian Postgraduate Award. I also wish to thank Professor Deane and The Smithsonian Institute for a sponsored period of research leave, and the Friends of the National Zoo, Washington D.C., and the Co-operative Research Centre for the Conservation and Management of Marsupials for financial support. I also thank the Marsupial CRC for permission to use the photographs of the tammar wallaby and the Rufous Hare-wallaby that appear with the section headings of this work.

Thank you to Professor Deane and Dr Gavan Harrison for supervision and direction throughout this study and together with Mark Young, for helpful comments on this manuscript.

A special thanks to Dr. Gavan Harrison for guidance, technical advice and a discerning taste in red wines.

I would also like to thank Professor Desmond Cooper of Macquarie University for advice in the early stages of this project and for ongoing support and Ron Claassens and Ann Moulard also of Macquarie University for expertise and assistance with animal management. Thank you to Dr. Peter Holz, Dr. Rosemary Booth, Dr. Rupert Baker and staff at the Healesville Animal Sanctuary (Melbourne) for advice and assistance with collection of animal tissue and blood samples; Dr. Ro McFarlane and staff at the Northern Territory Desert Park (Alice Springs) for useful discussions and the collection of animal samples, and Ross Boadle and staff at the Anatomical Pathology Laboratory (Electron Microscopy) at Westmead Hospital (Sydney) for training and assistance with electron microscopy. I also gratefully acknowledge the supply of species cross-reactive antibodies (anti-CD3, anti-CD5 and anti-CD79b) supplied by Dr. Margaret Jones of the Leukaemia Research Foundation (London) and the assistance provided by Caroline Wilson and Ann Peck with tissue processing and sectioning.
I acknowledge the contributions of Simone Coupe, Simon Flecknoe and Amy Slender, undergraduate students at the University of Western Sydney, who performed preliminary laboratory investigations under my direction during supervised research projects (innate cellular responses – SC & SF; immunohistochemistry - AS).

Thank you also to Associate Professor Thomas Millar of the University of Western Sydney for advice and much-valued assistance during the latter stages of my candidature, and for his willingness to pass on his computer expertise.

A very special thanks to Julie Old for the sanity-preserving ‘beverages’.

Finally, a thank you to Mark for his encouragement throughout.
# Table of Contents

**DECLARATION**  
**SUMMARY**  
**ACKNOWLEDGEMENTS**  
**TABLE OF CONTENTS**  
**LIST OF TABLES**  
**LIST OF FIGURES**  
**LIST OF ABBREVIATIONS**  

## SECTION I

**Marsupial Immunology and The Assessment of Immunological Capacity**

### CHAPTER ONE

**Marsupials and The Immune System**

1.1 Introduction  
1.2 The Division of Marsupials  
1.2.1 The Classification of Marsupials  
1.2.2 The Ecological Status of Australian Marsupials and the Need for a Macropod Marsupial Model  
1.3 The Significance of Evaluating Marsupial Cellular Responses  
1.4 Marsupials and Disease  
1.4.1 Diseases of Significance to Australian Marsupials - Susceptibility to intracellular pathogens  
1.5 Marsupial Immunology  
1.5.1 Overview  
1.5.2 Components of the Immune System  
1.5.3 The Origin and Function of Leukocytes  
1.5.4 The Cells of the Immune System  
1.5.5 Tissues of the Immune System  
1.5.6 Recognition of Antigen  
1.5.7 Molecules of the Immune System  
1.5.8 Immune Response to Mycobacterial Disease  
1.6 Project Rationale and Aims  
1.6.1 Hypothesis  
1.6.2 Project Aims

### CHAPTER TWO

**Materials and Methods**

2.1 Introduction  
2.2 Materials
2.2.1 Reagents and Consumables 47
2.2.2 Media 48
2.2.3 Stock Solutions 49
2.2.4 Animals 50
2.3 Methods 60
  2.3.1 Sample Collection 60
  2.3.2 Statistical Analyses 62
  2.3.3 Routine Identification of Leukocytes 63
  2.3.4 Isolation of Cells from Blood, Spleen and Lymph Nodes 65
  2.3.5 Phenotyping of Lymphocytes – Immunohistochemistry 71
  2.3.6 Acid-Fast Staining 74
  2.3.7 In Vitro Cellular Assays 75
  2.3.8 Molecular Characterisation of Immunoregulatory Molecules 89

SECTION II

The Tammar Wallaby (Macropus eugeni) ‘A Model Marsupial Species’ 102

  Introduction 104
  The Use of Model Species 104
  The Tammar Wallaby 104

CHAPTER THREE

Identification and Enumeration of Blood Leukocytes 106

  3.1 Introduction 106
  3.2 Results 107
    3.2.1 Separation of Individual Cell Populations 107
    3.2.2 Cell Classification and Enumeration 107
  3.3 Discussion 136
  3.4 Conclusion 151

CHAPTER FOUR

Isolation, Characterisation and Functional Capacity of Tammar Wallaby 153

Phagocytic Cells 153

  4.1 Introduction 153
  4.2 Results 154
    4.2.1 Isolation and Culture of Phagocytic Cells 154
  4.3 Discussion 191
  4.4 Conclusion 207

CHAPTER FIVE

The Capacity of Peripheral Blood Mononuclear Cells to Generate Responses In Vitro 208

  5.1 Introduction 208
5.1.1 Mitogens
5.1.2 Mitogen Stimulation of Marsupial Cells
5.1.3 Marsupial Cytokines

5.2 Results
5.2.1 Cell Isolation and Yield
5.2.2 Cell Culture
5.2.3 Proliferation Assays
5.2.4 Responses to Mitogens
5.2.5 Whole Blood Assays
5.2.6 Comparative Assays
5.2.7 Lymphocyte Responses to Cell Culture Supernatants
5.2.8 Molecules that Regulate the Immune Response

5.3 Discussion
5.4 Conclusions

SECTION III

Case Studies

Introduction
The Nature of the Study

CHAPTER SIX

Characterisation of Cells and Tissues

6.1 Introduction
6.1.1 Marsupial Peripheral Blood Cells
6.1.2 Marsupial Lymphoid Tissues
6.1.3 Mycobacteria

6.2 Results
6.2.1 Identification and Characterisation of the Peripheral Blood Leukocytes of the Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby
6.2.2 Identification and Characterisation of Tissues of the Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby

6.3 Discussion
6.4 Conclusion

CHAPTER SEVEN

Capacity to Generate an Immune Response

7.1 Introduction
7.2 Results
7.2.1 Isolation and Functional Capacity of Phagocytic Cells
7.2.2 Capacity to Generate A Non-Specific Response
7.2.3 Capacity to Generate A Specific Response
7.2.4 Molecules that Regulate the Immune Response
7.3 Discussion
7.4 Conclusion

SECTION IV
CHAPTER EIGHT
Overall Findings, Discussion and Future Directions

REFERENCES

APPENDIX ONE
PHA-driven Proliferation of Tammar Wallaby PBMC

APPENDIX TWO
Comparison of MTT Proliferation Results for Cells Isolated from Tammar Blood, Lymph Nodes and Spleen

APPENDIX THREE
Comparison of Tammar Wallaby IL-1β and IL-10 Sequences with those of Eutherian Mammals

APPENDIX FOUR
List of Genbank accession numbers and potoroo sample identities for partial Type I IFN sequences

APPENDIX FIVE
Representative Sample of Electrophoretogram

APPENDIX SIX
Publications and Conference Proceedings
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Stock solutions and working concentrations used in <em>in vitro</em> Cellular Assays</td>
<td>49</td>
</tr>
<tr>
<td>2.2</td>
<td>Tammar Wallaby Study Subjects</td>
<td>54</td>
</tr>
<tr>
<td>2.3</td>
<td>Potoroo Study Subjects</td>
<td>56</td>
</tr>
<tr>
<td>2.4</td>
<td>Rufous Hare-Wallaby Study Subjects</td>
<td>58</td>
</tr>
<tr>
<td>2.5</td>
<td>Antibody Specificity and Source</td>
<td>74</td>
</tr>
<tr>
<td>2.6</td>
<td>Oligonucleotide Primer Sequences for Cytokines and the Housekeeping Gene, G6PD</td>
<td>92</td>
</tr>
<tr>
<td>2.7</td>
<td>PCR Conditions for the Amplification of Partial cDNA sequences from Marsupial Tissues</td>
<td>93</td>
</tr>
<tr>
<td>3.1</td>
<td>Diameters of Tammar Wallaby Peripheral Blood Leukocytes</td>
<td>110</td>
</tr>
<tr>
<td>3.2</td>
<td>Tammar Wallaby Peripheral Blood Total Leukocyte Counts</td>
<td>123</td>
</tr>
<tr>
<td>3.3</td>
<td>Ratio of Lymphocytes to Neutrophils in Tammar Wallaby Peripheral Blood</td>
<td>124</td>
</tr>
<tr>
<td>3.4</td>
<td>Tammar Wallaby Monocyte, Eosinophil and Basophil Differential Counts</td>
<td>125</td>
</tr>
<tr>
<td>3.5</td>
<td>Tammar Wallaby Absolute Leukocyte Counts</td>
<td>126</td>
</tr>
<tr>
<td>3.6</td>
<td>Immunophenotype of Tammar Wallaby Peripheral Blood Lymphocytes</td>
<td>132</td>
</tr>
<tr>
<td>3.7</td>
<td>Total and Differential Leukocyte Counts of Selected Marsupial Species</td>
<td>148</td>
</tr>
<tr>
<td>4.1</td>
<td>Polarisation Responses of Tammar Wallaby Granulocytes</td>
<td>159</td>
</tr>
<tr>
<td>4.2</td>
<td>Migration of Tammar Wallaby Granulocytes</td>
<td>161</td>
</tr>
<tr>
<td>4.3</td>
<td>Respiratory Burst Indices of NBT-Treated Granulocytes</td>
<td>184</td>
</tr>
<tr>
<td>4.4</td>
<td>Cytochrome C Assay Optical Density Results for Stimulated Tammar Wallaby Granulocytes</td>
<td>185</td>
</tr>
<tr>
<td>4.5</td>
<td>Concentration of Nitrite Accumulated in Tammar Wallaby Adherent-Cell Culture Supernatants</td>
<td>186</td>
</tr>
<tr>
<td>4.6</td>
<td>Total Protein Content of Granule Extracts and Whole Cell Fractions from Tammar Wallaby Pooled Granulocytes</td>
<td>188</td>
</tr>
<tr>
<td>4.7</td>
<td>Bactericidal Activity of Granule and Whole Cell Fractions</td>
<td>189</td>
</tr>
<tr>
<td>5.1</td>
<td>Culture Conditions and Proliferation Responses of Mitogen Stimulated Marsupial Cells</td>
<td>212</td>
</tr>
<tr>
<td>5.2</td>
<td>Recovery of Lymphocyte Populations using Nylon Wool Columns</td>
<td>216</td>
</tr>
<tr>
<td>5.3</td>
<td>Survival of Tammar Wallaby PBMC in Different Culture Media</td>
<td>218</td>
</tr>
<tr>
<td>5.4</td>
<td>Comparison of Stimulation Indices (SI) of PHA-treated PBMCs using the MTT and [3H]-Thymidine Proliferation Assays</td>
<td>229</td>
</tr>
<tr>
<td>5.5</td>
<td>Proliferation Responses of Tammar Wallaby PBMC and Suspension Cells to PMA</td>
<td>239</td>
</tr>
<tr>
<td>5.6</td>
<td>Proliferation Responses of Whole Blood Cultures from the Tammar Wallaby</td>
<td>243</td>
</tr>
<tr>
<td>5.7</td>
<td>Tritiated Thymidine Incorporation of Mitogen-treated Human and Tammar Wallaby Whole Blood Cultures</td>
<td>244</td>
</tr>
<tr>
<td>5.8</td>
<td>Molecular Weights of Selected Cytokine Molecules</td>
<td>284</td>
</tr>
<tr>
<td>6.1</td>
<td>Diameters of the Peripheral Blood Leukocytes of the Long-footed Potoroo, the Long-nosed Potoroo and the Rufous Hare-wallaby</td>
<td>302</td>
</tr>
<tr>
<td>6.2</td>
<td>Differential Cell Counts of the Long-footed Potoroo and the Long-nosed Potoroo</td>
<td>304</td>
</tr>
<tr>
<td>6.3</td>
<td>Differential Cell Counts of the Rufous Hare-wallaby</td>
<td>305</td>
</tr>
<tr>
<td>6.4</td>
<td>Immunophenotyping of Mala and Potoroo Peripheral Blood Lymphocytes</td>
<td>306</td>
</tr>
<tr>
<td>6.5</td>
<td>Summary of the Detection of Acid-Fast Bacteria in Long-nosed Potoroo, Long-footed Potoroo and Mala Tissues</td>
<td>309</td>
</tr>
<tr>
<td>6.6</td>
<td>Summary of Key Histopathological Findings</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>7.1</td>
<td>Polarisation Responses of Granulocytes from the Long-footed Potoroo</td>
<td>354</td>
</tr>
<tr>
<td>7.2</td>
<td>NBT Responses of Mala and Potoroo Leukocytes</td>
<td>358</td>
</tr>
<tr>
<td>7.3</td>
<td>Respiratory Burst Indices of Treated Potoroo Granulocytes</td>
<td>362</td>
</tr>
<tr>
<td>7.4</td>
<td>Potoroo and Mala Whole Blood Mitogen-Driven Proliferation Responses</td>
<td>365</td>
</tr>
<tr>
<td>7.5</td>
<td>PHA-Driven Proliferation Responses of PBMC isolated from the Rufous Hare-wallaby, Long-nosed potoroo and Long-footed potoroo</td>
<td>366</td>
</tr>
<tr>
<td>7.6</td>
<td>Summary of Partial Cytokine cDNA Sequences Amplified from Macropod Species</td>
<td>377</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>Overview of the Development and Maturation of Cellular Immune Components</td>
<td>18</td>
</tr>
<tr>
<td>1.2</td>
<td>Development and Maturation of Myeloid Cells</td>
<td>21</td>
</tr>
<tr>
<td>2.1</td>
<td>Consensus Alignment of TNF-alpha Amino Acid Sequences</td>
<td>96</td>
</tr>
<tr>
<td>2.2</td>
<td>Consensus Alignment of TNF-alpha Nucleotide Sequences</td>
<td>97</td>
</tr>
<tr>
<td>2.3</td>
<td>Consensus Alignment of IL-1β Amino Acid Sequences</td>
<td>98</td>
</tr>
<tr>
<td>2.4</td>
<td>Consensus Alignment of IL-1β Nucleotide Sequences</td>
<td>99</td>
</tr>
<tr>
<td>2.5</td>
<td>Consensus Alignment of IL-10 Amino Acid Sequences</td>
<td>100</td>
</tr>
<tr>
<td>2.6</td>
<td>Consensus Alignment of IL-10 Nucleotide Sequences</td>
<td>101</td>
</tr>
<tr>
<td>3.1</td>
<td>Peripheral Blood Leukocytes of the Tammar Wallaby</td>
<td>108</td>
</tr>
<tr>
<td>3.2</td>
<td>Ultrastructure of Tammar Wallaby Mononuclear Cells</td>
<td>112</td>
</tr>
<tr>
<td>3.3</td>
<td>Ultrastructure of Tammar Wallaby Peripheral Blood Lymphocytes</td>
<td>113</td>
</tr>
<tr>
<td>3.4</td>
<td>Two Different Types of Tammar Wallaby Lymphocyte</td>
<td>114</td>
</tr>
<tr>
<td>3.5</td>
<td>Tammar Wallaby Peripheral Blood Mononuclear Cells</td>
<td>115</td>
</tr>
<tr>
<td>3.6</td>
<td>Ultrastructure of Mature Tammar Wallaby Granulocytes</td>
<td>119</td>
</tr>
<tr>
<td>3.7</td>
<td>Ultrastructure of a Neutrophilic Granulocyte</td>
<td>120</td>
</tr>
<tr>
<td>3.8</td>
<td>Ultrastructure of Tammar Wallaby Peripheral Blood Eosinophilic Granulocytes</td>
<td>121</td>
</tr>
<tr>
<td>3.9</td>
<td>Ultrastructure of Tammar Wallaby Granulocytes</td>
<td>122</td>
</tr>
<tr>
<td>3.10</td>
<td>Neutrophil and Lymphocyte Trends of Six Tammar Wallabies</td>
<td>127</td>
</tr>
<tr>
<td>3.11</td>
<td>Organisms found in the Blood of Apparently Healthy Tammar Wallabies</td>
<td>129</td>
</tr>
<tr>
<td>3.12</td>
<td>Cytochemical Localisation of Peroxidase in Tammar Wallaby Phagocytes</td>
<td>131</td>
</tr>
<tr>
<td>3.13</td>
<td>Tammar Wallaby Peripheral Blood Cells Stained with Anti-CD5 Antibody</td>
<td>135</td>
</tr>
<tr>
<td>4.1</td>
<td>Differential Interference Phase Contrast Micrographs of Tammar Wallaby</td>
<td>157</td>
</tr>
<tr>
<td>4.2</td>
<td>Polarisation Responses of Tammar Wallaby Granulocytes</td>
<td>158</td>
</tr>
<tr>
<td>4.3</td>
<td>Inverted Phase Micrograph of Adherent Cells Co-cultured with Lymphocytes</td>
<td>166</td>
</tr>
<tr>
<td>4.4</td>
<td>Phase Contrast Images of Tammar Adherent Cells in Culture</td>
<td>167</td>
</tr>
<tr>
<td>4.5</td>
<td>Tammar Wallaby Mononuclear Cells</td>
<td>168</td>
</tr>
<tr>
<td>4.6</td>
<td>Tammar Wallaby Multinucleated Giant Cells</td>
<td>169</td>
</tr>
<tr>
<td>4.7</td>
<td>Effect of Lipopolysaccharide Treatment on the Morphology of Tammar Wallaby</td>
<td>170</td>
</tr>
<tr>
<td>4.8</td>
<td>Phagocytosis of Latex Beads and Bacteria by Peripheral Blood Cells of the</td>
<td>174</td>
</tr>
<tr>
<td>4.9</td>
<td>Inverted Phase Micrographs of Adherent Cells Co-Cultured with Lymphocytes</td>
<td>175</td>
</tr>
<tr>
<td>4.10</td>
<td>Oxidative Burst Response of Tammar Wallaby Mononuclear Cells</td>
<td>178</td>
</tr>
<tr>
<td>4.11</td>
<td>NBT Staining of Tammar Wallaby Granulocytes</td>
<td>180</td>
</tr>
<tr>
<td>4.12</td>
<td>Representative Nitroblue Tetrazolium (NBT) Standard Curve</td>
<td>181</td>
</tr>
<tr>
<td>4.13</td>
<td>Representative Standard Curve for Nitrite Determination in Culture Supernatants</td>
<td>187</td>
</tr>
<tr>
<td>5.1</td>
<td>Tammar Wallaby PBMC in Culture</td>
<td>220</td>
</tr>
<tr>
<td>5.2</td>
<td>Tammar Wallaby PBMC after Culture in Serum-free Media for 5 Days</td>
<td>221</td>
</tr>
<tr>
<td>5.3</td>
<td>Colony Morphology of Peripheral Blood Mononuclear Cells</td>
<td>223</td>
</tr>
<tr>
<td>5.4</td>
<td>MTT Standard Curve for Suspension Assays</td>
<td>226</td>
</tr>
<tr>
<td>5.5</td>
<td>MTT Standard Curve</td>
<td>227</td>
</tr>
<tr>
<td>5.6</td>
<td>Optical Density of Control and PHA-treated Lymphocytes using the MTT Assay</td>
<td>230</td>
</tr>
<tr>
<td>5.7</td>
<td>Relationship Between the [3H]-thymidine and MTT Incorporation Assays</td>
<td>230</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>5.8</td>
<td>MTT Suspension Cell Proliferation Assay in RPMI + 5% Pooled Tammar</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>5.9</td>
<td>Proliferation of Tammar Wallaby PBMC in response to PHA</td>
<td>234</td>
</tr>
<tr>
<td>5.10</td>
<td>Time Course for Proliferation of Tammar Wallaby PBMC in response to</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td></td>
</tr>
<tr>
<td>5.11</td>
<td>Proliferation of Tammar wallaby PBMC in response to Concanavalin A</td>
<td>236</td>
</tr>
<tr>
<td>5.12</td>
<td>PWM-Driven Proliferation of Tammar Wallaby PBMC in RPMI + 10% FBS</td>
<td>238</td>
</tr>
<tr>
<td>5.13</td>
<td>PWM-Driven Proliferation of Tammar Wallaby PBMC in Serum-free Media</td>
<td>238</td>
</tr>
<tr>
<td>5.14</td>
<td>Time Course for PWM-Driven Proliferation of Tammar Wallaby PBMC</td>
<td>239</td>
</tr>
<tr>
<td>5.15</td>
<td>Proliferation Response of Tammar Wallaby PBMC to Lipopolysaccharide</td>
<td>242</td>
</tr>
<tr>
<td>5.16</td>
<td>Proliferation of Human PBMC Cultured with both Serum-Free and Serum-</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>supplemented Media</td>
<td></td>
</tr>
<tr>
<td>5.17</td>
<td>Proliferation Response of Nylon Wool Separated Cells to PHA in Serum-</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>free Medium</td>
<td></td>
</tr>
<tr>
<td>5.18</td>
<td>Proliferation of Lymph Node Cells to Mitogens in Serum-Supplemented</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>and Serum-Free Media</td>
<td></td>
</tr>
<tr>
<td>5.19</td>
<td>Proliferation of Lymph Node Cells to Mitogens in Serum-Free Media</td>
<td>250</td>
</tr>
<tr>
<td>5.20</td>
<td>Proliferation of Mitogen-Stimulated Spleen Cells for 24 and 48 hours</td>
<td>252</td>
</tr>
<tr>
<td>5.21</td>
<td>Proliferation of Mitogen-Stimulated Tammar Wallaby Spleen Cells</td>
<td>252</td>
</tr>
<tr>
<td>5.22</td>
<td>Inverted Phase Micrograph of Tammar Wallaby Suspension Cells after</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>Treatment with Mitogen-Stimulated Cell Supernatant</td>
<td></td>
</tr>
<tr>
<td>5.23</td>
<td>Proliferation Response of Tammar Wallaby PBMC to Supernatants</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>Harvested from Cultured Mononuclear Cells</td>
<td></td>
</tr>
<tr>
<td>5.24</td>
<td>2% Agarose in TBE gel of IL-1β products</td>
<td>260</td>
</tr>
<tr>
<td>5.25</td>
<td>2% Agarose in TBE gel of IL-10 products</td>
<td>261</td>
</tr>
<tr>
<td>5.26</td>
<td>Comparison of IL-1β and IL-10 partial cDNA Sequences from M. eugenii</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>and T. vulpecula</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Peripheral Blood Leukocytes of the Long-footed Potoroo and the Long-</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>nosed Potoroo</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>Peripheral Blood Leukocytes of the Rufous Hare-wallaby</td>
<td>300</td>
</tr>
<tr>
<td>6.3</td>
<td>Electron Micrographs of the Peripheral Blood Leukocytes of the Rufous</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>Hare-wallaby</td>
<td></td>
</tr>
<tr>
<td>6.4</td>
<td>Immunocytochemical Staining of the Lymphocytes of the Long-footed</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>Potoroo and the Rufous Hare-wallaby</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>Acid-fast Staining of Mala and Potoroo Tissues</td>
<td>310</td>
</tr>
<tr>
<td>6.6</td>
<td>Histology of Mala Lymph Nodes (H and E)</td>
<td>314</td>
</tr>
<tr>
<td>6.7</td>
<td>Histology of Potoroo Lymph Nodes (H and E)</td>
<td>315</td>
</tr>
<tr>
<td>6.8</td>
<td>Immunohistological Staining of Long-footed Potoroo and Mala Lymph</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>Nodes</td>
<td></td>
</tr>
<tr>
<td>6.9</td>
<td>Histology of Mala and Potoroo Spleen (H and E)</td>
<td>319</td>
</tr>
<tr>
<td>6.10</td>
<td>Immunohistological Staining of Mala Spleen</td>
<td>320</td>
</tr>
<tr>
<td>6.11</td>
<td>Histology and Immunohistology of Tissue from Mala Small Intestines</td>
<td>322</td>
</tr>
<tr>
<td>6.12</td>
<td>Histology of Potoroo Lung (H and E)</td>
<td>324</td>
</tr>
<tr>
<td>6.13</td>
<td>Histology of Mala Lung (H and E)</td>
<td>325</td>
</tr>
<tr>
<td>6.14</td>
<td>Histology of Mala Liver and Adrenal Gland Lesions (H and E)</td>
<td>328</td>
</tr>
<tr>
<td>6.15</td>
<td>Liver, Spleen and Skin Lesions (H and E)</td>
<td>329</td>
</tr>
<tr>
<td>7.1</td>
<td>Inverted Phase Micrograph of Mala Macrophages</td>
<td>354</td>
</tr>
<tr>
<td>7.2</td>
<td>Differential Interference Phase Contrast Micrographs of Long-footed</td>
<td>355</td>
</tr>
<tr>
<td></td>
<td>Potoroo Granulocytes</td>
<td></td>
</tr>
<tr>
<td>7.3</td>
<td>Relative Biomass of Migrating Granulocytes isolated from the Long-</td>
<td>356</td>
</tr>
<tr>
<td></td>
<td>footed Potoroo</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Phagocytosis of Inert and Biological Particles by Potoroo Cells</td>
<td>357</td>
<td></td>
</tr>
<tr>
<td>NBT Responses of Leukocytes from Long-nosed and Long-footed Potoroos</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>NBT Response of Granulocytes of the Brush-tailed Bettong</td>
<td>361</td>
<td></td>
</tr>
<tr>
<td>NBT Deposition in Granulocytes of Small Wallaby Species and Two Monotremes</td>
<td>364</td>
<td></td>
</tr>
<tr>
<td>Culture Morphology of Long-nosed Potoroo PBMC in Different Media</td>
<td>367</td>
<td></td>
</tr>
<tr>
<td>Culture of Long-nosed Potoroo PBMCs in Different Media</td>
<td>367</td>
<td></td>
</tr>
<tr>
<td>2% Agarose in TBE Gel of Long-footed potoroo Type I IFN PCR Product Clones</td>
<td>370</td>
<td></td>
</tr>
<tr>
<td>Type I IFN Maximum Parsimony Tree</td>
<td>371</td>
<td></td>
</tr>
<tr>
<td>Comparison of TNF-α cDNA Sequences from Macropods and <em>T. vulpecula</em></td>
<td>374</td>
<td></td>
</tr>
<tr>
<td>Ethidium Bromide Stained 2% Agarose in TBE Gel of Eco R1 Digested Plasmid Preparations Prepared from Mala and Long-footed potoroo cDNA</td>
<td>375</td>
<td></td>
</tr>
<tr>
<td>Comparison of IL-10 cDNA Sequences from Macropods and <em>T. vulpecula</em></td>
<td>376</td>
<td></td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMVRT</td>
<td>Avian Myeloblastosis Virus Reverse Transcriptase enzyme</td>
</tr>
<tr>
<td>ANGIS</td>
<td>Australian National Genomic Information System</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance unit</td>
</tr>
<tr>
<td>BALI</td>
<td>bronchus associated lymphoid tissue</td>
</tr>
<tr>
<td>BCG</td>
<td>bacille Calmette-Guerin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine tetrachloride</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>dT</td>
<td>deoxythymidine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>g</td>
<td>unit of gravity</td>
</tr>
<tr>
<td>GALN</td>
<td>gut associated lymph node</td>
</tr>
<tr>
<td>GALT</td>
<td>gut associated lymphoid tissue</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
</tr>
<tr>
<td>G6PD</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>³H</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>H and E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution without phenol red</td>
</tr>
<tr>
<td>HBSS-</td>
<td>Hanks' Balanced Salt Solution without phenol red, calcium and magnesium ions</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K</td>
<td>thousand</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LAF</td>
<td>lymphocyte activating factor</td>
</tr>
</tbody>
</table>
LB  Luria Bertani
LBAgar Luria broth agar
LCSFM lymphocyte conditioned serum-free media
LPS lipopolysaccharide
LSA lymphocyte stimulation assay
MAC Mycobacterium avium complex
MALT mucosa associated lymphoid tissue
MBP major basic protein
MgCl₂ magnesium chloride
MHC major histocompatibility complex
μm micrometre
milliQ milliequivalent
mg milligram
mL millilitre
mins minutes
MLC mixed lymphocyte culture
MLR mixed leukocyte reaction
mM millimolar
MOPS 3-(N-Morpholino) propanesulphonic acid
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW molecular weight
MWCO molecular weight cut-off
MYA million years ago
n number in group or study
NBT nitroblue tetrazolium chloride
ND not determined
ND not detectable
ng nanogram
nm nanometre
nM nanomolar (10⁻⁹ moles per litre)
NO nitric oxide
NO₂⁻ nitrite
NOS nitric oxide synthase
OD optical density
P probability
PAGE polyacrylamide gel electrophoresis
PBL peripheral blood lymphocyte
PBMC peripheral blood mononuclear cell
PBS phosphate-buffered saline
PCR polymerase chain reaction
PHA phytohaemagglutinin
PMA phorbyl myristate acetate
PMN polymorphonuclear leukocytes
PPD Purified Protein Derivative
PWM pokeweed mitogen
PY pouch young
QBSF® Quality Biologicals serum-free media
RACE rapid amplification of cDNA ends
RBI respiratory burst index
RBC red blood cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNI</td>
<td>reactive nitrogen intermediate</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediate</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute cell culture media</td>
</tr>
<tr>
<td>RPI</td>
<td>relative proliferation index</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SOZ</td>
<td>serum-opsonised zymosan</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA buffer</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TWS</td>
<td>tammar wallaby serum</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre (10^{-6} litre)</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre (10^{-6} metre)</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar (10^{-9} moles per litre)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
SECTION I

Marsupial Immunology
and
The Assessment of
Immunological Capacity
CHAPTER ONE

Marsupials and The Immune System

1.1 Introduction

Free-ranging marsupials can be difficult to study in their native habitat and, as a result, captive populations are used to learn more about the immunobiology of these animals. However, captive animals are exposed to a variety of conditions not experienced in their natural environment. These include, but are not limited to, such factors as more strictly controlled diets, smaller and more prescribed living areas, stresses due to confinement and isolation from social groups. Some species of animals held in captivity demonstrate diminished reproductive capacity and are often bred from very few founder individuals which may contribute to the accumulation of undesirable genetic traits within the captive group (Luikart et al., 1997). These and other less defined factors appear to have resulted in captive marsupial populations being particularly vulnerable to the effects of disease (Dubey et al., 1988; Montali et al., 1998; Buddle and Young, 2000). At present, there is a dearth of information available describing the nature of the immunological responses of these animals. The present study was undertaken in an effort to provide this fundamental data for one family of marsupials, the kangaroos and wallabies (superfamily Macropodidae). The work contained herein describes some of the basic in vitro immune responses of macropodid marsupials in health and disease and provides a set of benchmarks for future immunological studies.

1.2 The Division of Marsupials

Metatherians, more commonly known as marsupials, are one of the three groups of animals that comprise the Class Mammalia. The other two groups include the prototherian (monotreme) and the eutherian (placental) mammals (Novacek, 1992; Graves, 1996). Reproductive strategies differ amongst these three groups and prototherian mammals, represented by the echidna (family Tachyglossidae) and the platypus (family Ornithorhynchidae), are clearly distinguished from the other mammalian groups by their characteristic egg-laying ability (Strahan, 1998).
It is estimated that the separation of Eutheria and Metatheria took place from as early as approximately 150 million years ago (MYA) to as late as 75 MYA (Air et al., 1971; Hope et al., 1990; Novacek, 1992; Janke et al., 1997; Kirsch et al., 1997; Belov et al., 2001; Killian et al., 2001) and differences between these two phylogenetically distinct lineages are chiefly related to their reproductive strategies. Eutherian mammals give birth to well-developed young that are nourished in-utero by a placenta throughout gestation. In general, metatherians have a comparatively short gestation time that results in the birth of young with immature neurological and immunological systems (Tyndale-Biscoe and Janssens, 1988). These young typically develop in an external pouch during a long lactation period that may last for up to 300 days in some species (Lillegraven et al., 1987). Thus, most marsupials are at a less advanced stage of development at birth than any eutherian mammal (Tyndale-Biscoe, 1973).

1.2.1 The Classification of Marsupials

Marsupials have played a central role in mammalian evolutionary studies and there remains much debate about the phylogenetic relationships that exist within this group (Springer et al., 1994; Kirsch et al., 1997; Palma and Spotorno, 1999). It is estimated that there are 280 living marsupial species with approximately two-thirds of this number inhabiting Australia and New Guinea (Strahan, 1998). With a few exceptions, the remaining species are from the opossum family (Didelphidae) and are found in South America. Together with physiological attributes, this distinct geographical separation has resulted in the classification of American and Australian marsupials into two separate cohorts: the Ameridelphia and the Australidelphia (Springer et al., 1994). Within the cohort Australidelphia, the subclass Marsupialia comprises mammals that are born with immature physiological systems. The development and maturation of these animals usually occurs when the young is ensconced in a pouch whilst attached to a teat. This group is further divided into four orders: the Dasyuromorpha that are carnivores or insect-eaters; the Peramelemorpha, the bandicoots and bilbies; the Notoryctemorpha, the marsupial mole, and the Diprotodontia, which comprises koalas, wombats, possums, kangaroos and wallabies (Strahan, 1998).

Early marsupial classification relied strongly on morphological attributes such as dentition and pedal characteristics (Tyndale-Biscoe, 1973) and this classification system pervades much of the present day literature. Species with only one pair of
functional incisors in the lower jaw and fused second and third toes on the hindfeet are assigned to the *Diprotodontia*. Those that possess many incisors in both upper and lower jaws with separated digits are assigned to the *Polyprotodontia* and include the Australian carnivores, bandicoots and American opossums (Strahan, 1998). More recently, these classifications have been challenged with information gained from serological, biochemical and molecular studies (reviewed in Novacek, 1992, Springer *et al.*, 1994 and Kirsch *et al.*, 1997) and a number of species have been reassigned to new or different taxa. There is however, broad acceptance of *Diprotodontia* as a marsupial order (Kirsch *et al.*, 1997; Strahan, 1998).

The *Diprotodontia* is divided into suborders and superfamilies, a classification system that accommodates the diversity found within this large group. The suborders Vombatiformes (koalas and wombats), Phalangeriformes (possums) and Macropodiformes (kangaroos) contain the majority of the most commonly recognised marsupials. The family *Macropodidae*, within the *Macropodoidea*, consists of 56 species that include 45 found in Australia. Approximately 24 million years ago, the macropodid subfamilies *Potoroinae* and *Macropodinae* arose within this group (Kirsch *et al.*, 1997). The *Potoroinae*, with eight Australian species that include the potoroo, bettongs and rat-kangaroos, are small macropods that generally weigh less than three kilograms. Their diet mainly consists of tubers, fungi and insects that they acquire through digging with their forelimbs. The second group, the *Macropodinae*, with 37 species that include the wallabies, kangaroos, pademelons and the quokka, are large macropods that range from 3-85 kg and are predominantly grazers (Cronin, 1991; Strahan, 1998; Mate *et al.*, 1999). Based on deoxyribonucleic acid (DNA) hybridisation data, the potoroine and macropodine kangaroos are more similar to each other than to any other diprotodontian families (Kirsch *et al.*, 1997).

1.2.2 The Ecological Status of Australian Marsupials and the Need for a Macropod Marsupial Model

The *Marsupialia* contains a large number of living species whose status range from ‘extinct in the wild’ through to over-abundant (Maxwell *et al.*, 1996; Cowan and Tyndale-Biscoe, 1997). Within the family *Macropodidae*, the tammar wallaby and the Rufous Hare-wallaby are classified as ‘extinct in the wild’, the Long-footed potoroo as endangered, and the Long-nosed potoroo as vulnerable (Hilton-Taylor,
2000). Other macropod species such as the Eastern and Western Grey kangaroos (*M. giganteus* and *M. fuliginosus*) and the Red kangaroo (*M. rufus*) are considered pests and are the subjects of controversial harvesting programs (Cowan and Tyndale-Biscoe, 1997). Along with other members of the *Marsupialia*, macropod marsupials are kept as part of captive groups such as those found in research colonies and fauna exhibits.

In order to address the problems of population control and management associated with both pest and endangered species, a number of immunoreproductive strategies are being developed that exploit the basic mechanisms of the immune response (Mate *et al.*, 1998). These measures include immunocontraceptive techniques that may assist with humane population reduction as well as assisted reproductive technologies that attempt to ensure the successful production of offspring from endangered animals. These approaches clearly require knowledge and understanding of marsupial biology including the fundamental processes that govern the marsupial immune system. A suitable model species in which to develop protocols that enable measurement of immunological responses is the tammar wallaby (*Macropus eugenii*). Whilst this species is extinct in the wild, it breeds well in captivity (Renfree, 1981a) and has been used as a subject for immunoreproductive studies (Mate *et al.*, 1998).

In Australia, whilst some individual species have bred in abundance in particular locations, the overall numbers of marsupial species have suffered a decline since the arrival of European settlers over 200 years ago (Szabo, 1995). Some twenty species of native mammals are thought to have become extinct during this time including the Tasmanian tiger (*Thylacinus cynocephalus*), the Central Hare-wallaby (*Lagorchestes asomatus*) and the Broad-faced potoroo (*Potorous platyops*) (Wilson and Friend, 1999). In the past decade, of the known 142 living Australian marsupials, 61 were under threat of extinction.

Extinctions may be caused by a number of factors that include habitat degradation and predation by exotic animals (Wilson and Friend, 1999). Similarly, the effects of disease, particularly in species that are already considered vulnerable, may also play a large part in the reduction of population size.
1.3 The Significance of Evaluating Marsupial Cellular Responses

In eutherian mammals, susceptibility to mycobacterial infection and the onset of chronic disease is generally associated with deficiencies in acquired immunological responses, specifically, cell-mediated immunity (Carpenter et al., 1997). Non-specific cellular defence strategies have also been demonstrated to play a role in the establishment of initial infection (Brown et al., 1987). In order to identify the factors involved in the apparent susceptibility of captive marsupial species to intracellular pathogens (see below), there is a need to evaluate the functional capacity of marsupial immune cells. In this study, this has been undertaken with particular reference to their ability to combat invasion by intracellular pathogens such as the *Mycobacteriaceae*.

*Mycobacterium avium* causes clinically important infections in human patients with marked deficiencies in their immune system (Hartmann et al., 2001). In marsupials, infections with mycobacterial species have mainly been reported in captive animals, especially those that are vulnerable in terms of population numbers (Phelan, 1996; Montali et al., 1998). *M. avium* has been documented in the Brush-tailed bettong (*Bettongia penicillata*; Richardson and Read, 1986), the Matchie’s tree-kangaroo (*Dendrolagus matschiei*; Joslin, 1990, Montali et al., 1998) and the Long-footed potoroo (*Potorous longipes*; Phelan, 1996). A number of other atypical mycobacterial infections have also been reported in captive marsupial species (reviewed by Buddle and Young, 2000).

1.4 Marsupials and Disease

Marsupials are reported to be affected by disease in captivity, and an understanding of factors that contribute to this susceptibility is essential for their effective management (Bettiol et al., 1998). In the wild, species such as the brushtail possum in New Zealand may serve as disease vectors for livestock (Cowan and Tyndale-Biscoe, 1997) and potentially have a significant effect on the economy. Furthermore, the close proximity of some animals to human populations, particularly tourists, as occurs with quokka (*Setonix brachyurus*) populations on Rottnest island, may promote the transfer of zoonotic infections such as Salmonellosis (Munday, 1972). The lack of available knowledge regarding both the incidence of disease and characterisation of immune responses of metatherian mammals limits our ability to treat infections and to
understand the underlying factors that enable pathogens to successfully colonise metatherian hosts.

1.4.1 Diseases of Significance to Australian Marsupials - Susceptibility to intracellular pathogens

Marsupials are reported to have problems dealing with intracellular pathogenic invasion (see below). Infections with Herpesvirus, *Toxoplasma gondii*, *Salmonellae* and *Mycobacteriaceae* have caused significant losses in captive and free-ranging marsupial populations. Successful colonisation of the host by intracellular pathogens such as bacteria and protists rely on the ability of these organisms to survive and replicate within the host cell. The following section outlines the incidence of some of the intracellular pathogens that affect marsupial populations.

1.4.1.1 Viral Disease

Exposure to Herpesvirus is common in animals of the family *Macropididae* and is reported to cause fatalities in a number of species in this group (Wilks *et al.*, 1981). Macropod herpesviruses have been associated with disease and fatalities in captive populations of Australian marsupials (Reddacliff *et al.*, 1997; Smith and Whalley, 1998). Furthermore, higher proportions of animals kept in captivity possess neutralising antibodies to these organisms when compared with their free-ranging counterparts (Guliani *et al.*, 1999). It has been proposed that conditions in captivity such as stress and crowding may lead to higher viral transmission rates and latent viral reactivation (Webber and Whalley, 1978).

Herpesviruses have been isolated from a number of macropod species including the parma wallaby (*M. parma*) (Finnie *et al.*, 1976), the dorcopsis wallaby (*Dorcopsis muelleri luctuosa*) and the quokka (*Setonix brachyurus*) (Wilks *et al.*, 1981). Given the widespread nature of these infections, reactivation of latent viral infections may serve to compromise the ability of marsupials to respond effectively to other disease agents.

1.4.1.2 Salmonellosis

Salmonellosis is a disease caused by species of the intracellular bacterial genus *Salmonellae*. In macropods, this bacterium causes debilitating disease and has been
linked to the deaths of the Red kangaroo (Barker et al. 1963), the quokka (Hart et al., 1985), and the koala (Munday, 1978). However, large proportions of animals carry significant burdens of these organisms with no apparent clinical effect (Munday, 1988). *Salmonella* bacteria are more commonly isolated from marsupials than from eutherian mammals and therefore these animals have the potential to serve as reservoirs for human disease.

### 1.4.1.3 Toxoplasmosis

It is widely accepted that marsupials are highly susceptible to infection with the intracellular protozoan parasite *T. gondii* (Lynch et al., 1993b; Innes, 1997). Australian marsupials such as the Black-faced kangaroo (*M. fuliginosus*), the koala, the Bennett’s wallaby (*M. rufogriseus*), the Tasmanian pademelon (*Thylogale billardierii*) and the Eastern Barred Bandicoot (*Parameles gunnii*) are all vulnerable to infection with *T. gondii* (Dubey et al., 1988; Canfield et al., 1990; Hartley et al., 1990; Johnson et al., 1989; Booth, 1994). In the development of Toxoplasmosis, protective immunity and the initial response to invasion by *T. gondii* are controlled in immunocompetent individuals by the cell-mediated immune response (Innes, 1997). Thus, the susceptibility of metatherian mammals to this pathogen implies a defect in this aspect of their immune system.

Acute infection with *T. gondii* in marsupial species often results in fatality (Innes, 1997) which is consistent with responses observed in immunocompromised eutherian mammals affected with this pathogen. With the exception of the New World Monkeys, marsupials are amongst the most susceptible of warm-blooded animals to *T. gondii* (Canfield et al., 1990; Innes, 1997). In evolutionary terms, this group of mammals had relatively recent contact with members of the *felidae*, which function as primary hosts for *T. gondii*. Consequently, contact with cats in the wild or in captive situations such as zoos has resulted in infection and a subsequent inability to control the progression of pathogenic disease (Innes, 1997). Since a number of marsupial species developed in isolation from introduced pathogens, the role of lymphoid tissue antigen naivety may also be an important consideration in the susceptibility of marsupials to another family of debilitating intracellular pathogens, the *Mycobacteriaceae*. 
1.4.1.4 Mycobacterial Disease

Awareness of mycobacterial infections in the general community is often limited to the affects of *M. tuberculosis*, the causative agent of human tuberculosis (TB). Approximately one third of the human population is infected with TB and between five and 10 percent of this number progress to active disease (Anderson, 2001; Chackerian *et al.*, 2001). In the majority of cases, the human immune system is able to effectively control this pathogen. Vaccination has been widely used to limit the spread of this form of mycobacterial infection and the current TB vaccine, *Mycobacterium bovis* bacille Calmette-Guerin (BCG), is still widely used even though it is no longer considered effective in providing widespread prevention (Anderson, 2001). Infections with the non-tuberculosis mycobacteria such as *M. avium* and *M. intracellulare*, whilst increasing in recent times, are generally only reported in humans when the host is immunocompromised (Ohga *et al.*, 1997).

1.4.1.4.1 Atypical Mycobacteria

Atypical mycobacterial species infect a range of animals and cause pulmonary disease in humans that is similar to that caused by *M. tuberculosis* (Prescott *et al.*, 1993; Bermudez *et al.*, 1997). The *M. avium-M. intracellulare* (MAI) complex leads to disseminated disease in up to 50% of humans that have Acquired Immune Deficiency Syndrome (AIDS) (Bermudez *et al.*, 1997; Tomioka *et al.*, 1997). *M. avium* was first identified in humans in 1943 in association with a pre-existing case of lung disease (Ashford *et al.*, 2001). Organisms within this group can cause disease in both healthy and immunocompromised individuals, although it is more prevalent amongst the immune depressed. Captive marsupial species are considered to be susceptible to mycobacterial disease and this susceptibility has led to the assumption that these animals are themselves immunocompromised or possess an immune system that is less sophisticated than their eutherian relatives (Montali *et al.*, 1998).

Mycobacteria are known to successfully colonise a number of marsupial species but the susceptibility of these animals to infection appears to vary. While the Australian brushtail possum is highly susceptible to *M. bovis* (Buddle *et al.*, 1994), *M. avium* appears to be the principal cause of mycobacterial disease in macropods (Peet and Dickson, 1982).
Atypical, or non-tuberculous mycobacteria, are ubiquitous in nature and are found in both soil and water (Escuyer et al., 1996). They are able to survive unfavourable conditions for lengthy periods and are spread throughout the environment by avian vectors, which increases their potential to infect a broad spectrum of animals. Whilst it is clear that captive marsupials are prone to infection with this genera (reviewed by Buddle and Young, 2000), few clinical studies have achieved successful identification of the primary infection source. Using Pulsed Field Gel Electrophoresis and Restriction-Fragment Length Polymorphism analysis of bacterial isolates, Tree kangaroos held at The National Zoological Park (Virginia, USA) were found to be infected by a range of mycobacterial strains from a number of different sources from within the animals’ enclosures (Montali et al., 1998). In Australia, Gaynor and Friend (1990) described the infection of two captive numbats (Myrmecobius fasciatus); a female with *M. intracellulare* and a male with *M. chelonae*. Both *M. intracellulare* and *M. fortuitum* were isolated from habitat soil but neither of these isolates was proven to be responsible for the original infections. Schultz et al. (1996) and Obendorf (1983) documented infections of koalas with *M. ulcerans*, a toxin-producing strain of mycobacteria not often reported to infect other marsupial species. The causative organism was also believed to be environmentally acquired.

Recovery of marsupials from atypical mycobacterial infections is rarely reported, although treatment of the two captive numbats with various antibiotic regimes reported above resulted in successful recovery of the male infected with *M. chelonae* and poor response and subsequent death of the female infected with *M. intracellulare* (Gaynor and Friend, 1990). Accounts of clinically normal animals that mount positive skin tests for mycobacterial antigens are also rare, however asymptomatic Goodfellow’s tree kangaroos (*D. goodfellowi*) (Joslin, 1990) and Long-footed potoroos (Phelan, 1996) tested positive for *M. avium* and *M. fortuitum* and for *M. avium* respectively.

### 1.5 Marsupial Immunology

#### 1.5.1 Overview

The neonatal marsupial develops in an ‘external’ pouch that is populated with a variety of microbes that include some species recognised as opportunistic pathogens in mammals (Yadav et al., 1972; Charlick et al., 1981; Old and Deane, 1998). The
protective mechanisms available to the young animal have thus been the subject of much interest. Many of the early marsupial immunological studies focussed on characterising the mechanisms available to the newborn in order to ensure their survival in an antigen-laden environment (reviewed by Deane and Cooper, 1988). Immunological strategies available to pouch young include prenatal transfer of maternally derived antibodies via the yolk sac (Deane et al., 1990) and postnatal transfer via the colostrum and milk (Yadav, 1971; Samples et al., 1986; Deane et al., 1990). Cellular components also found in the milk feeding young quokkas (Cockson and McNeice, 1980), tammars (Young et al., 1997) and koalas (Young and Deane, 2001) may represent another maternal immunoprotective strategy. However, whilst these cells are commonly found in eutherian milk (Newby et al., 1981), their role in the immunological protection of young marsupials has yet to be defined.

Studies of marsupial immunology to date suggest that the development, maturation and function of the marsupial immune system is similar but not identical to eutherian mammals (Ashman et al., 1975; Stone et al., 1997 and 1998; Old and Deane, 2000). It is now well established that, at least at the anatomical level, marsupials have histologically similar lymphoid tissues to other vertebrates (Block, 1964; Basden et al., 1997; Old and Deane, 2001). However, examination of the identity, distribution and function of the different cell populations within these tissues is still in its infancy. Results from a number of immunohistological studies using species cross-reactive antibodies against cell-surface markers suggest that lymphocytes of the marsupial immune system possess similar surface antigens to those of eutherian mammals (Coutinho et al., 1995; Hemsley et al., 1995; Baker et al., 1999; Cisternas and Armati, 2000; Old and Deane, 2001).

In vitro functional studies of cells isolated from a variety of marsupials have generally focussed on the capacity of lymphocytes to respond to immunological challenge with plant mitogens that act as polyclonal activators (Ashman et al., 1976; Infante et al., 1991; Wilkinson et al., 1992b; Buddle et al., 1994). These studies have been supplemented to a very small degree by studies of antigen stimulation (Buddle et al., 1994; Wilkinson et al., 1994). Cellular immune responses that demonstrate the ability to reject skin allografts and to yield positive skin reaction to antigen sensitisation have also been demonstrated in a number of different species (Rowlands, 1976; Buddle et
al., 1997). However, as demonstrated in studies of the North American opossum (Didelphis virginiana) and the grey, short-tailed opossum (Monodelphis domestica), all metatherian species studied to date appear to possess weak or non-existent mixed lymphocyte responses (MLR) (Fox et al., 1976; Stone et al., 1998). The significance of this is yet to be established but these results may in part be explained by differences in the numbers and distribution of Major Histocompatibility Complex (MHC) antigens, the molecules thought to be responsible for MLR responses, since such variations have been noted in a number of mammalian species (Davis and Hamilton, 1998).

The early study of the marsupial humoral immune system focussed on antibody responses to both particulate and soluble antigens in the North American opossum (La Via et al., 1963, Taylor and Burrell, 1968; Rowlands and Dudley, 1969; Rowlands, 1970) and on antigen responses and identification of immunoglobulin molecules in the Australian quokka (Thomas et al., 1972; Bell et al., 1974). The molecular characterisation of immunoglobulin molecules (reviewed by Miller and Belov, 2000) and more recent studies of mucosal immune responses (Khalil et al., 2001) confirms earlier assumptions that whilst there are differences in the magnitude and timing of marsupial antibody responses, the complexity of the humoral response to antigen is similar to that found in other vertebrate mammals (Shearer et al., 1995).

As outlined above, the majority of marsupial immunological studies have concentrated on the specific or acquired arm of the immune response. The primary or innate response has been largely neglected with the exception of a small number of reports on complement proteins (Wirtz and Westfall, 1967; Koppenheffer et al., 1998) and one study of the acute phase response (Richardson et al., 1998). Studies of phagocytic cell populations are limited to an incidental report of the in vivo behaviour of splenic macrophages in D. virginiana (Marx et al., 1971) and to one investigation of the chemotactic properties of possum lung macrophages (Moriarty and Thomas, 1986). In general, innate immune mechanisms are presumed to be similar to those of eutherian mammals but this presumption remains unsubstantiated and unexplored.

In the past decade, a number of immunoregulatory molecules have also been reported as part of the marsupial immune portfolio. Cytokines, glycoproteins that have proven
roles in the modulation of immune responses in eutherians (Mire-Sluis and Thorpe, 1998), have been identified using molecular techniques in the brushtail possum and the tammar wallaby (reviewed by Harrison and Wedlock, 2000). Verification of the biological activity of recombinant proteins produced from two of these molecules, Tumour Necrosis Factor alpha (TNF-α) and Interleukin 1 beta (IL-1β), has been established in the brushtail possum by thymocyte stimulation assays (Wedlock et al., 1999a and 1999b). Others have proposed that IL-1 and IL-2 are present in the supernatants of stimulated lymphocyte cultures after assessment of these preparations for bioactive properties (Brozek and Ley, 1991; Wilkinson et al., 1992b). However, the identity of these molecules has yet to be proven.

Although the study of marsupial immunology is still in its infancy when compared with what is known about the eutherian immune system, marsupials appear to possess the capacity to mount an array of immunological defences capable of dealing with antigenic assault. However, much is still to be learned about the fundamental immunological mechanisms available to metatherian mammals and this requires knowledge of both the structure and function of cells, tissues and soluble components that defend these animals against pathogenic invasion.

1.5.2 Components of the Immune System

The major functions of the mammalian immune system enable the body to discriminate its own cells from that of a foreign organism and to protect the host against foreign invaders (Benjamini et al., 2000). Immediate responses to invasion by pathogens occur via the non-specific branch of the immune system, which acts rapidly to produce various global antimicrobial effectors. These may be both cellular, as in the case of Natural Killer (NK) cells, or soluble, examples of which include the complement proteins, cytokines such as TNF and antimicrobial peptides such as the defensins (Fearon and Locksley, 1996). Aside from the provision of immediate immune protection to the host, the innate immune response also provides a link with the slower acting, but more antigen-specific, acquired immune response. This response is activated by molecules that recruit specialised cells to the site of injury (chemokines) or by presentation of antigens to other immune cells that possess a memory for the generation of a response to the antigen in question (Fearon and Locksley, 1996; Abbas et al., 1997).
The immune system functions to recognise and eradicate foreign material from the host. In all mammals studied to date, the immune response to these antigens consists of both soluble and cellular components. Soluble components may be cytotoxic or regulatory in their nature and are released by cells after activation by the presence of antigens. A number of different cell types produce these soluble molecules and also deal directly with antigen by isolating the foreign material within phagocytic vacuoles or by direct lysis (Abbas et al., 1997; Roitt et al., 1998).

The innate immune response is raised immediately the host detects the presence of foreign material in the form of micro-organisms or tissue damage caused by injury or inflammation. When foreign organisms invade, myeloid cells such as monocytes, macrophages and granulocytes deal non-specifically with this incursion by releasing chemical mediators such as superoxide radicals and nitric oxide to eradicate the threat (Janeway et al., 1999). These cells are also phagocytic and are able to remove the pathogen from the circulation and degrade it within a phagocytic vacuole that contains this process. Granulocytes also possess a number of different granule populations that contain an array of biochemical defence molecules such as myeloperoxidase, lactoferrin and a family of small cationic peptides, the defensins (Borregaard and Cowland, 1997).

If antigen either survives or escapes these processes, cells of the lymphoid system are activated. Mature bone marrow derived lymphocytes (B cells) produce antibodies that are able to bind with antigen and effect their removal. Thymus derived lymphocytes (T cells) and macrophages deal directly with pathogens and also produce cytokines that regulate the type and duration of the specific immune response (Goldsby et al., 2000). In eutherian mammals, both of these systems work simultaneously and independently to eradicate the invading organism. In metatherian mammals, the interaction of these systems has yet to be investigated since the specific immune response has received the most research focus (see earlier discussion) and much of the workings of the innate system have been presumed. This presumption has remained largely unchallenged due to the similarity between these primitive responses amongst most members of the animal kingdom (Borregaard et al., 2000).
The following section provides an account of the innate and acquired immunological strategies available to mammals with particular reference to the current knowledge of metatherian immune function.

1.5.3 The Origin and Function of Leukocytes

In mammals, cells of the immune system found in peripheral blood are derived from a common multipotent precursor cell known as a stem cell (see Figure 1.1). Both erythrocytes and leukocytes are formed in the bone marrow in the process of haematopoiesis from this founder cell line (Banks, 1981). Leukocytes that develop in this process can be divided into three principal categories: granulocytes, monocytes and lymphocytes. Monocytes circulate in the peripheral blood and migrate to the tissue beds where they mature and become macrophages. The granulocytes (neutrophils, eosinophils and basophils) are also found in the circulatory system and are highly motile cells that are usually the first of the immune cells to encounter antigen (Cassatella, 1995).

In eutherian mammals such as humans and mice, both the monocyte and granulocyte cell populations arise from a myeloid stem cell that differentiates into the two separate cell lineages under the influence of cytokines known collectively as colony stimulating factors. The production of these growth factors determines the maturation pattern of cells based on the requirements of the host (Hoffman et al, 2000). Myeloid progenitor cells develop through a series of stages and ultimately give rise to both red and white cell forms. Erythrocytes and platelets (produced from megakaryocytes) originate from this precursor cell as do the granulocytes and monocyte-derived cell lines. Neutrophils, eosinophils, basophils and monocytes differentiate from this line via a granulocyte-monocyte precursor cell (see Figure 1.2). Both neutrophils and monocyte/macrophage cell populations form the professional phagocytic arm of cellular defence.

The lymphoid progenitor stem cell gives rise to cell populations that are responsible for the specific or acquired responses of the immune system. All lymphocytes are derived from bone marrow precursor cells and differentiate within the primary lymphoid tissues. Precursor T cells travel to the thymus where they develop into thymocytes before release into the circulation. In mammals, B cells develop in the
bone marrow (Fulcher and Basten, 1997). These lymphoid cells migrate to the peripheral or secondary lymphoid tissue such as the spleen and lymph nodes where they are in a prime position to encounter antigen delivered by the circulatory or lymphatic systems.

In humans, haematopoiesis begins in blood islands of the embryonic yolk sac (Sharma et al., 1997) and then continues in the liver and spleen of the foetus. With increasing age, production of leukocytes is transferred to the bone marrow, where white blood cells develop and ultimately enter the circulation (Burkitt et al., 1993). This process is similar in metatherian mammals where haematopoetic activity transfers from the liver to the bone marrow within the first two weeks after birth (Basden et al., 1996). In the absence of evidence to the contrary, the similarity in structure, appearance and development of the haematopoetic cells and tissues of marsupials suggests that the differentiation and maturation of leukocytes parallels that of eutherian mammals.
Figure 1.1: Overview of the Development and Maturation of Cellular Immune Components. Adapted from Benjamini et al. (2000) and Goldsby et al. (2000). *Some cytokine production.
1.5.4 The Cells of the Immune System

There are five main types of leukocytes found in the blood and lymphatic systems of mammals (Banks, 1981). Cells that arise from the myeloid lineage, the monocyte family and the granulocytes, are principally involved in the innate immune response and deal with antigen invasion with an array of responses that include phagocytosis and the production of cytotoxic biochemicals. The tissue-based macrophage cell populations also play a significant role in the specific response to pathogens by processing antigens and presenting them to lymphoid cells. Along with lymphoid cells, macrophages produce immunoregulatory molecules, the cytokines, which control the precise nature of the response to particular antigens (Thomson, 1998).

Lymphocytes such as the T and B cells are responsible for the cell-mediated and humoral responses that arise after antigen has successfully breached the primary immune defences. T cells secrete cytokines, e.g. Interleukin-2, and collaborate with macrophages in cell-mediated responses (Paul and Seder, 1994). B cells may also produce cytokines, e.g. Interleukin-12, but are principally involved in the humoral response and are responsible for the synthesis of antibodies that assist with the clearance of antigen. There are five major classes of immunoglobulin or antibody molecules found in human plasma, IgM, IgG, IgA, IgE and IgD (Benjamini et al., 2000). All of these molecules share a similarity of form that includes two structurally different sections, the heavy chain and the light chain. The heavy chain determines the class of Ig and is linked to one of two known light chains, the kappa and lambda chains. A region of both the heavy and light chain forms the variable region of the antibody and forms a binding site specific for recognition of a unique antigen (Roitt et al., 1998). The constant section of the molecule is responsible for the effector functions of antibodies which may include the coating of invading antigens thus targeting them for destruction or the direct neutralisation of antigen by forming immune complexes that are either soluble or are cleared by phagocytosis (Janeway et al., 1999).

1.5.4.1 Phagocytes

The migration of activated leukocytes to the site of injury or infection involves a sequence of events that begins with the response to a chemoattractant followed by migration of reactive cells to the affected site (Wilkinson and Haston, 1988). The first
of the phagocyte populations to arrive at the infection site are neutrophils whose dominant function is to deal directly with the invading antigen by ingestion followed by chemical destruction of the foreign material (Morel et al., 1991). Macrophages usually follow within 72 hours of the initial infection. Like neutrophils, macrophages are directly responsible for the eradication of antigen but also serve the dual purpose of removing dead and dying neutrophils from the tissues (Burkitt et al., 1993).

Since they are amongst the first of the cell populations to contact antigen, phagocytes play an important immunological role in the protection of mammals against bacterial and parasitic invasion. Resident macrophages and circulating monocytes and polymorphonuclear cells produce chemical cytotoxins and phagocytose material as part of the innate immune response (Badwey and Karnovsky, 1980). Both of these myeloid cell lines are able to ingest particulate matter as a preliminary step in the destruction of pathogens. Once degraded into peptide fragments, macrophages and dendritic cells are able to present the processed antigen to lymphoid cells (Abbas et al., 1997).

As part of the initiation phase of phagocytosis, antibodies coat the surface of microbes and orient themselves so that one specific region, the Fragment crystallisable (Fc), of the antibody molecule is exposed. Phagocytic cells possess Fc receptors that are then able to bind to the coated microbe. Other types of receptors on these cells recognise opsonins such as complement (a group of plasma proteins with immunological functions against extracellular bacteria) and oligosaccharides that can directly mediate antigen recognition (van der Laan et al., 1997). As well as dealing directly with pathogens, macrophages also perform a housekeeping role by ingesting dead and damaged cells and removing them from the tissue beds (Alberts et al., 1989; Roitt, 1991).
Figure 1.2: Development and Maturation of Myeloid Cells. Adapted from Burkitt et al. (1993) and Goldsby et al. (2000).
1.5.4.1.1 Granulocytes – Polymorphonuclear Phagocytes

The granulocytes are the principal effector cells of the innate response and are easily recognised by their multilobed nuclei, distinctive cytoplasmic granules and characteristic staining properties evident when using routine light microscopy techniques (Smith and Wood, 1992).

Neutrophils

Neutrophils are produced by haematopoiesis in the bone marrow, where they are the most abundant nucleated cell type. They are short-lived cells that in health are only released into the bloodstream in their mature form. They circulate for approximately 12 hours before they either migrate into the tissue beds or respond to chemotactic signals released by cells at the site of an infection (Squier et al., 1995). Neutrophils play a significant role in the control and eradication of infectious agents since they are the first of the leukocyte cell populations to respond to antigenic assault. At such times, these cells are rapidly released from the bone marrow in increased numbers which may result in the presence of immature forms circulating in the blood. Along with the increase in total white blood cell numbers (leukocytosis), the presence of immature neutrophils circulating in the peripheral blood pool is symptomatic of a state of infection (Hoffman et al., 2000).

In eutherian mammals, the granules contained within neutrophils are classified according to size and composition. Primary or azurophilic granules are usually the most dense of the granules and contain lytic enzymes such as peroxidase and lysozyme (Root and Cohen, 1981) and the alpha defensins, antimicrobial peptides that exert their activity by permeabilising cell membranes (Tang et al., 1999). Secondary granules are smaller and may also contain lysozyme as well as collagenase and lactoferrin. In some mammalian species, a third larger granule has been identified that contains lactoferrin as well as bactericidal cationic polypeptides (Roy et al., 1997). Neutrophilic granules carry out their cytotoxic functions by fusion with phagosomes that contain the ingested debris, followed by release of their contents into the newly formed structure so that the antigenic material is destroyed (Benjamini et al., 2000).

The polymorphonuclear neutrophils of marsupials possess similar morphology and staining properties to those of eutherian mammals (Ponder et al., 1929; Lewis et al.,
1968; Hawkey et al., 1982). There is however, a trend for the nuclei of these cells to be hyperlobulated when compared with other mammalian species (Hawkey, 1977). Ultrastructural studies of these cells are limited to one study of the koala (Canfield and Dickens, 1982) and another on the fat-tailed dunnart (*Sminthopsis crassicaudata*; Haynes and Skidmore, 1991) that confirm the presence of heterogeneous granules within the cytoplasm of these cells. The nature and function of these granules remains undescribed.

Eosinophils

Along with neutrophils, eosinophils are mature motile leukocytes that are amongst the first cells to be recruited to injury sites. Their specialised functions include provision of host defence against helminthic parasites and response to allergens (Root and Cohen, 1981). As part of the phagocytic cell population, they contain oxidative and non-oxidative defences such as peroxidases and proteases but these occur at less than 10% of the levels found in neutrophils (Weller, 1994). Where pathogens are too large to be phagocytosed, the major defensive role of eosinophils is mediated by the secretion of granule contents onto the surface of the invading pathogen. This assault causes damage to the parasitic membrane and hence destruction of the pathogen (Root and Cohen, 1981).

Eosinophils are easily identified from other blood leukocytes by the presence of large, coarse, cationic cytoplasmic granules. Mature eosinophils have a segmented nucleus that is commonly bilobed. In most mammals studied to date, eosinophilic granules contain a crystalloid core that is principally composed of major basic protein (MBP) (Schalm et al., 1975). These crystalloid-containing granules, called ‘specific’ granules, characterise mature eosinophils. Acidic histological stains such as eosin have an affinity for the proteins found within these granules and are used to identify these cell types. Unlike polymorphonuclear neutrophils that are thought to be end-stage effector cells, mature eosinophils possess centrioles and remain biosynthetically active (Zucker-Franklin et al., 1981).

Eosinophils have been reported in marsupial haematological studies (Ponder et al., 1929; Barbour, 1972; Hawkey et al., 1982) and are often associated with parasitic burdens (Spencer and Speare, 1992). Differences in granule size and morphology are described for some species (Canfield and Dickens, 1982) but the significance of these
differences is yet to be established. Ultrastructural analysis of marsupial eosinophils is again limited to the one study of koala leukocytes that confirmed the presence of crystalloid granule inclusions in cells from this species (Canfield and Dickens, 1982).

Basophils and Mast Cells

Basophils are the least common of the blood leukocytes. They were, until recently, thought to be nonphagocytic, granulocytic cells that function only in particular allergic responses (Goldsby et al., 2000). Their functionality has received renewed attention and together with mast cells, their immunological role is being redefined with reports of both the production of cytokines and phagocytic behaviour (Abraham and Arock, 1998). In general though, only fundamental characterisation of these cell populations has been achieved. In eutherian mammals, basophilic granules contain approximately 25% of the amount of major basic protein as eosinophils and smaller amounts of three other cationic proteins (Weller, 1994). Histamine is the major constituent of large, coarse granules that are generally stained blue with base-loving dyes (Burkitt et al., 1993). Histamine, a vasodilator, attracts leukocytes to the site of inflammation and is released after IgE binding. Up to 20 different proteases have also been identified in some eutherian mast cells (Chan et al., 2001).

Basophils usually possess a U or S-shape nucleus that has a number of constrictions (Banks, 1981). These cells have been identified in accounts of marsupial blood leukocytes but are generally present in very low numbers and are rarely present in routine examinations. Basophils with morphology and staining characteristics consistent with the appearance of eutherian cells have been reported in the Long-nosed potoroo (P. tridactylus; Moore and Gillespie, 1968), the brushtail possum (Barbour, 1972), the koala (Canfield and Dickens, 1982), the allied rock-wallaby (Petrogale assimilis; Spencer and Speare, 1992) and the eastern quoll (Dasyurus viverrinus; Canfield, 1998).

The tissue equivalent of the basophil is the mast cell. In eutherians, mast cells, like basophils, are involved in hypersensitivity reactions but have also been demonstrated to produce cytokines and mediate proliferative and suppressive immune responses (Abraham and Arock, 1998). Two different forms of mast cells have been identified in the lymph nodes of five species of South American marsupials. Like those identified by Haynes (1991) in the fat-tailed dunnart, these cells varied in their
location, granule size and composition (Chiarini-Garcia and Pereira, 1999). Functional studies have yet to prove whether these cells play the same role as their eutherian equivalents in metatherian immune function.

### 1.5.4.1.2 Monocytes, Macrophages and Dendritic Cells - Mononuclear Phagocytes

#### Monocytes and Macrophages

Monocytes are released from the bone marrow into the blood before they migrate to the tissue beds and develop into macrophages (Smith and Wood, 1992). As part of the innate immune branch, macrophages are not antigen specific but do play an important role in the specific immune response by presenting processed antigen to T lymphocytes (Benjamini et al., 2000).

In health, monocytes comprise approximately 5% of the blood leukocytes. They are recognised by the presence of a characteristic horseshoe-shaped nucleus and clearly differentiated cytoplasm when inspected by routine microscopy techniques. Monocytes have been identified in metatherian haematological studies and appear to possess similar morphological characteristics to other mammals. However, reports of little or no enzyme activity in cells of the brushtail possum (Barbour, 1972) and grey short-tailed opossum (Armstrong and Ferguson, 1997) suggest a difference in number and composition of the granules in these cells compared with eutherian mammals.

#### Dendritic Cells

Dendritic cells are accessory cells that are involved in the initiation of T lymphocyte responses to protein antigens (Paquette et al., 1998). They express high levels of both class II MHC molecules and members of the co-stimulatory B7 family. They are therefore more effective antigen-presenting cells (APCs) than macrophages and B cells. Moreover, some populations of dendritic cells do not need to be activated before they perform this function (Goldsby et al., 2000). A subpopulation of these cells, follicular dendritic cells, do not express MHC class II molecules and therefore do not act as APCs. They are located in lymph nodes in the B cell rich areas and are thought to play a role in B cell activation independent of T cell help. Both immature and antigen-activated dendritic cells are found in the peripheral blood of humans en route to their target tissues (O’Doherty et al., 1994).
1.5.4.1.3 Phagocytosis and the Production of Chemical Mediators

Once at the site of antigen or tissue damage, granulocytes are able to generate an armoury of antimicrobial responses. These may be divided into oxidative and non-oxidative defences. Oxidative responses involve the generation of toxic oxygen radicals, nitric oxide and chloramines. Non-oxidative responses include the release of small cationic proteins called defensins that lyse bacterial cells by creating ion-channels in the cell membrane (Evans and Harmon, 1995). There have been no reports of the biochemical effector molecules present in marsupial phagocytes, so the following section describes what is currently known about the phagocytic responses and associated biochemical reactions of eutherian cells.

Oxidative Defences

In the event of immune challenge, reactive oxygen intermediates (ROI) may be involved in intracellular signalling and destruction of the invading pathogen. They may also cause injury to host cells and tissues in the event of a local inflammatory response. Oxidative burst products are used for killing of phagocytosed pathogens and for the extracellular destruction of other cells (Root and Cohen, 1981). Along with the consumption of increased quantities of glucose and oxygen, stimulated phagocytes undergoing the respiratory burst response produce superoxide anions and hydrogen peroxide. In these series of reactions, superoxide is generated by single electron reduction of molecular oxygen by NADPH oxidase (Pick, 1986) which is present in ‘professional’ phagocytic cells such as neutrophils, monocytes, macrophages and eosinophils. Within the endocytic vacuole, this enzyme generates superoxide anions and hydrogen peroxide. The production of hydrogen peroxide results from the combination of two superoxide radicals or by dismutation by the enzyme superoxide dismutase and can subsequently lead to the production of hydroxyl free radicals and hypochlorous acid (Bokoch and Knaus, 1994). All of these oxidative burst products assist in the destruction of phagocytosed material.

Nitric Oxide

Nitric oxide is a small, reactive, signalling molecule that is produced in mammals by various cell types including eosinophils, macrophages, smooth muscle cells and epithelial cells (Yates, 2001). It interacts with molecular oxygen to form nitrite and nitrates. Reactive nitrogen intermediates (RNI) include nitric oxide (NO) and the oxidised forms NO₂ (nitrate) and NO₃ (nitrate). Nitric oxide is a potent cytotoxic
agent and may be released from cells or generated within vacuoles. It can block cellular respiration by complexing with iron in electron transport proteins (Taylor-Robinson, 1997) or combine with the superoxide anion to form oxidants such as the peroxynitrite anion. This molecule is involved in the nitrosation of proteins and nucleic acids and may also mediate lipid peroxidation. Mammalian cells produce nitric oxide from L-arginine via nitric oxide synthase enzymes. There are various forms of these enzymes but the Type II or calcium and calmodulin-independent NO synthases (iNOS) are responsible for NO synthesis in response to endotoxin lipopolysaccharides and to pathophysiological changes associated with endotoxic shock (Yates, 2001). NO has been shown to play a role in host defence by direct cytotoxic properties and by mediation of inflammation. This molecule may also influence the profile of immunoregulatory molecules by promoting decreased levels of Interferon-gamma (IFN-γ) (Yates, 2001).

As part of the arsenal of microbicidal molecules available to the host, NO influences both resistance and susceptibility to disease. It is known to play a role in the pathogenicity of intracellular pathogens and is responsible for direct destruction of organisms such as *T. gondii* (Taylor-Robinson, 1997) and reduced resistance and reactivation of disease associated with *M. avium* (Mohan *et al.*, 2001).

**Non-oxidative defences**

Antimicrobial Proteins

Innate molecular mechanisms such as antimicrobial cationic peptides are present as part of the host defence components of plants, insects and mammals (Borregaard *et al.*, 2000). These molecules are amphiphatic cationic molecules that insert into the phospholipid bilayers of microorganisms and form lytic pores. This mechanism involves the displacement of magnesium ions (Mg²⁺) from lipopolysaccharide (LPS) and the insertion of the peptide into the bacterial membrane. Antimicrobial peptides have been identified in the neutrophil granules of humans (Weiss *et al.*, 1978), rabbits (Selsted *et al.*, 1984), cows (Selsted *et al.*, 1993) and pigs (Alberdi *et al.*, 1995) but have yet to be identified in any metatherian species.

1.5.4.2 Lymphocytes

The antigen-specific or acquired response of the immune system is mediated by lymphocytes. These cells both recognise and respond to foreign antigens and produce
products that regulate antigen-specific responses. Lymphocytes can be found in various tissues and organs of the body where they perform a number of direct and indirect immunological functions. In order to optimise their ability to contact antigen, lymphocytes are constantly migrating throughout the body through lymphoid and non-lymphoid organs (Westermann and Bode, 1999). Antigen-presenting cells, found in the T cell areas of lymphoid organs, present antigen to these naïve T cells, which results in their activation and proliferation within the tissue beds. Activated T cells then return to the circulatory pool (Westermann and Bode, 1999). There are two major classes of lymphocytes, B cells and T cells, that specifically recognise and respond to antigens. B cells produce immunoglobulins or antibodies and T cells become activated or sensitised to perform several cell-mediated functions (Zucker-Franklin et al., 1981).

1.5.4.2.1 T cells
After production in the bone marrow, T lymphocytes migrate to the thymus where they develop into immunocompetent cells. T cells form the major population of lymphocytes in peripheral blood (Hunt, 1987) and perform a number of distinctly different roles in the immune response. T-helper (T_{h}) cells assist B cells in the production of antibody and promote the destruction of intracellular pathogens by interaction with macrophages. Cytotoxic T (T_{c}) cells also assist with the eradication of intracellular organisms by direct lysis of the infected host cell (Roitt et al., 1998). T lymphocytes recognise antigen with a T cell receptor (TCR) after it is presented on the surface of an APC in association with MHC molecules. The T cell receptor is structurally similar to the B cell receptors or immunoglobulin molecules of B cells.

1.5.4.2.2 B cells
B lymphocytes mature into antibody producing plasma cells after stimulation by antigen. At the light microscope level, B cells and T cells cannot be differentiated unless the B cell is actively synthesising antibody. In such cases, B cells can be distinguished by their eccentrically placed nucleus, abundant cytoplasm and a distinctive perinuclear halo. Plasma cells are believed to be terminally differentiated cells with little or no capacity for mitotic division (Zucker-Franklin et al., 1981).
1.5.5 Tissues of the Immune System

A large number of the immune cells that are involved in antigen recognition and response are found in the circulation but the majority of these cells are localised in tissues and organs that trap and concentrate antigenic material. These areas are collectively known as secondary lymphoid tissues and include the lymph nodes, spleen and mucosa-associated lymphoid tissues (MALT). As well as these organised lymphoid structures, scattered aggregates of lymphocytes may also be found surrounding blood vessels and within tissues where direct contact with antigen is possible (Burkitt et al., 1993).

1.5.5.1 Spleen

Both blood and lymph-borne antigens are processed through secondary lymphoid tissues, with the spleen being the major site of immune responses to blood-borne antigens. In eutherian mammals, this organ houses the single largest site of secondary lymphoid tissue (Hunt, 1987) and has a unique structural arrangement that consists of an irregular pattern of areas called white and red pulp. The white pulp of the spleen consists of concentrated lymphoid tissue that includes the T cell rich periarteriolar lymphoid sheaths (PALS) and lymphoid follicles that contain germinal centres (Gartner and Hiatt, 1994). In mammals, the B cell rich follicles are surrounded by a marginal zone, which consists of a boundary of lymphocytes and macrophages. Splenic arterioles end in vascular sinusoids that together with erythrocytes, macrophages, dendritic cells, lymphocytes and plasma cells, form the red pulp. Sinusoids eventually drain into the splenic vein, which carries blood from the spleen into the circulatory system. Macrophages play an important role within the spleen, since apart from their role in antigen presentation within this organ, they also function as blood filters by removing old erythrocytes and foreign material (Burkitt et al., 1993).

1.5.5.2 Lymph Nodes

Lymph nodes are involved in responses to antigens carried in lymphatic fluid and are bean-shaped capsule-enclosed organs that are found lining lymphatic vessels where the opportunity for exposure to antigens is maximal. Antigens are transported to these areas after entering via cutaneous routes and are also collected along with cells from interstitial spaces (Abbas et al., 1997). The superficial cortex of the node contains groups of lymphocytes arranged in circular patterns called lymphoid follicles. When
treated with routine histological stains, these follicles are classified as primary or secondary, dependent on the absence or presence of a central pale area known as a germinal centre (Burkitt et al., 1993). Germinal centres are the sites of B lymphocyte proliferation and their presence generally signifies that a humoral immune response has recently occurred or is in progress. Within the node, B cells are also found in large numbers within the medullary regions. T lymphocytes are most numerous in the deep cortical zone or paracortex, which is located beneath and around the lymphoid follicles (Gartner and Hiatt, 1994).

1.5.5.3 Mucosal-Associated Lymphoid Tissue (MALT)

The mucosal immune system contains lymphoid tissue that is situated close to the site of antigen exposure (Lugton, 1999). Included in these areas are the gut associated lymphoid tissue (GALT) found lining the digestive system, and the bronchus associated lymphoid tissue (BALT) within the respiratory system. These areas are commonly seeded with lymphoid nodules that contain cells that facilitate antigen presentation (Gartner and Hiatt, 1994).

GALT consists of structurally organised lymphoid regions such as Peyer’s patches and mesenteric lymph nodes. Lymphoid cells can also be found scattered within the gut tissue. In eutherians, lymphocytes play an important role in immunological protection of the lung. These cells may be found in an organised framework known as BALT. Where this immune tissue develops, lymphoid cells are present in large numbers with interstitial lymphocytes in the lung of humans and rats present in numbers that rival that found in peripheral blood (Pabst and Binns, 1994). The major sites of the mucosal immune system, the lungs (through the respiratory system) and the gut (through the digestive system) are also commonly seeded with discrete collections of lymphoid cells called lymphoid nodules.

1.5.5.4 Location of Lymphocytes within Lymphoid Tissues

Antigen presentation to immunoincompetent T cells generally occurs in areas of lymphoid tissues that contain dense regions of T lymphocytes (Westermann and Bode, 1999). The paracortex of lymph nodes, the periarteriolar lymphoid sheaths (PALS) of the spleen and the T-cell compartments of Peyer’s patches, are areas where helper (CD4+) T cells respond to antigens presented on APCs in association with MHC Class II molecules (Bell and Sparshott, 1997). The lymphoid regions that
contain the highest numbers of B cells include the Peyer’s patches of the gut tissue (Hunt, 1987) and the marginal zone of splenic lymphoid follicles (Burkitt et al., 1993).

1.5.6 Recognition of Antigen

In eutherian mammals, antigen is recognised by cell receptor molecules found on the surface of both B and T cells. B cell receptors may also be secreted as circulating immunoglobulin (Ig) molecules. T cell receptor (TCR) molecules are cell bound and recognise antigen only in association with another class of antigen receptor molecule, the Major Histocompatibility Complex (MHC) glycoprotein (Sprent, 1991). CD4^+ helper T cells recognise antigen bound to MHC Class II molecules and CD8^+ cytotoxic T cells recognise antigen bound to MHC Class I molecules (Yeager and Hughes, 1999).

The detection and expression of a number of antigen recognition molecules within the marsupial genome has been confirmed using standard molecular techniques, and thus far, it appears that the relationship of antigen-receptor molecules and lymphocytes is similar in these species to those of eutherian mammals. Complementary deoxyribonucleic acid (cDNA) sequences for the TCR alpha and beta chains have been cloned from the brushtail possum, the tammar wallaby (Zuccolotto et al., 2000) and the South American opossum (M. domestica; Baker et al., 2001). The cDNA for the CD3 epsilon (CD3e) chain, a component of the complex associated with signal transduction via the TCR, has also been cloned in the tammar wallaby (Old et al., 2001). The lymphocyte development molecules, recombination activating gene-1 and terminal deoxynucleotidyl transferase have been cloned and sequenced in M. domestica (Miller and Rosenberg, 1997; Guth et al., 1998) and the sequences for a variety of immunoglobulin molecules have also been described (see 1.5.7.3).

1.5.6.1 The Major Histocompatibility Complex (MHC)

MHC molecules are glycoproteins that assist with the initiation of an immune response by binding to antigens and presenting them on the cell surface. MHC Class I molecules are found on all nucleated body cells and present foreign proteins generated by intracellular pathogens such as viruses (Weenink and Gautam, 1997). These molecules are principally responsible for transplantation reactions and have been identified in a number of marsupial species. MHC Class I molecules have been
reported in the red-necked wallaby (*M. rufogriseus*; *Mayer et al.*, 1993), the koala (*Houlden et al.*, 1996), the brushtail possum (*Lam et al.*, 2001), and the South American opossum (*Stone et al.*, 1987 and 1997; *Miska and Miller*, 1999). Whilst work in this area is still very exploratory, results to date suggest that human and marsupial MHC genes have arisen from different gene origins (*Houlden et al.*, 1996).

MHC Class II molecules are found on the surface of specialised antigen presenting cells such as dendritic cells, macrophages and some B cells. Extracellular pathogens are usually processed by macrophages and presented as peptides in association with Class II MHC glycoproteins (*Weenink and Gautam*, 1997). MHC Class II genes have been identified in the red-necked wallaby (*Schneider et al.*, 1991), the tammar wallaby (*McKenzie and Cooper*, 1994; *Slade et al.*, 1994), the South American opossum (*O’hUigin et al.*, 1998; *Stone et al.*, 1999) and the brushtail possum (*Lam et al.*, 2001).

### 1.5.6.2 Cell-Mediated Immune Responses

Cell mediated immune responses are mediated by lymphocytes from the T cell lineage that are directly or indirectly involved in the response to antigen (*Roitt et al.*, 1998).

#### 1.5.6.2.1 Mixed Lymphocyte Culture (MLC) Responses

An MLC reaction is an *in vitro* assessment of the proliferation of T-cells from responder lymphocytes from one animal to histocompatibility antigens on the surface of mononuclear cells of another (donor) animal (*Rose et al.*, 1992). Thus, measures of the variability within the MHC Class II family are demonstrated using this technique. When tammar wallaby MLC responses were studied in an investigation of the immunology of reproduction, these responses appeared to parallel those of eutherian mammals (*Walker and Tyndale-Biscoe*, 1978) although in most other studies of marsupial MLC responses, results have suggested that MLC responses are reduced or non-existent when compared with most eutherian mammals (*Fox et al.*, 1976; *Wilkinson et al.*, 1992a; *Stone et al.*, 1998). These poor MLC responses have led to a great deal of speculation regarding the functioning of the marsupial cell-mediated immune system. Suggestions of impairment in T cell function linked to the absence of MHC Class II polymorphism or atypical T cell receptors (*Fox et al.*, 1976; *McKenzie and Cooper*, 1994; *Stone et al.*, 1998) are accepted yet disputable paradigms. Since allogeneic lymphocyte stimulation may still be positive when test
subjects possess T-cell deficiencies (Rose et al., 1992), these apparently reduced responses require further investigation.

In addition to MLC responses and the functions outlined in 1.5.4.2.1, T cells also secrete immunoregulatory molecules, the cytokines, which control the magnitude and timing of the antigen response. In the delayed-type hypersensitivity inflammatory reaction, these molecules are responsible for the activation and recruitment of monocytes and macrophages to the site of antigen exposure (Benjamini et al., 2000).

1.5.6.2.2 Delayed Type Hypersensitivity (DTH) Response

Once activated by antigen, T cells are able to produce cytokines that activate other cell populations, particularly phagocytes, and cause their migration to the affected site. This reaction is not immediate and generally occurs within one to two days after the immune challenge (Roitt et al., 1998). Once at the antigen site, macrophages begin destruction of the foreign organism. If the antigen is recognised by the host and T cells are activated, this response may cause damage to the surrounding tissue and this response can be identified by its characteristic pathology. This response is generally assumed to mean that prior exposure to the pathogen in question has occurred.

In macropod marsupials, this test often proves inconclusive when assessment of subclinical infection is undertaken. This is demonstrated in Matschie’s tree kangaroos afflicted with mycobacterial osteomyelitis where testing using Avian and Bovine Purified Protein Derivative (PPD) Tuberculin and Avian Old Tuberculin resulted in redness at the injection site, but no obvious DTH reaction (Joslin, 1990). Brushtail possums also show variable responses to skin testing dependent on the antigen in question (Buddle et al., 1994). Skin testing of BCG-immunised possums with varying doses of bovine PPD results in positive skin test responses, but requires much higher doses than that of guinea pigs (Harris, 1995). When immunised with heat-killed M. tuberculosis or BCG, possums produce weak DTH skin reactions. Similarly, when tested for prior exposure to M. tuberculosis, D. virginiana also displayed weak skin test results when compared with those of the rabbit (Taylor and Burrell, 1968).
Other attempts to use tuberculin testing in tree kangaroos and potoroos have resulted in inconsistent responses that appear to be of limited diagnostic value (Phelan, 1996; Montali et al., 1998). Animals do not often test positive, even when later shown to be infected with atypical mycobacteria. This lack of DTH response is similar to that seen in aggressive forms of tuberculosis in eutherian mammals and is also typical of that displayed by immunocompromised hosts who show an increased susceptibility to atypical infection. Whilst depressed immune functionality may account for some of these variable responses, it is also possible that the properties of the bacterium, rather than the host, are a major determining factor in the immune responses raised in these skin tests.

Assessment of the cell-mediated response in vitro is used in both eutherian and metatherian immunological studies as a correlate for the capacity of T cells to respond in vivo. In these test regimes, isolated lymphocytes are stimulated with the specific antigen under study, or with polyclonal activators such as plant mitogens that cause non-specific activation of lymphocyte populations within the cultures (Sharon, 1983).

1.5.6.2.3 Mitogen-driven responses
In eutherian mammals, the ability of plant lectins to induce broad lymphocyte responses is used to monitor immune competence in vitro (Kristensen et al., 1982b). They have similar stimulatory properties to that of antibodies and bacterial products (Janossy and Greaves, 1971) and have been used historically in the study of marsupial immune responses.

The mitogens that are most often used in in vitro studies include Concanavalin A (Con A), Phytohaemagglutinin (PHA), Pokeweed mitogen (PWM) and Lipopolysaccharide (LPS). Con A and PHA are both T cell activators and PWM is able to stimulate both T and B cells. The lipid portion of LPS, a constituent of the walls of gram negative bacteria, is a dedicated B cell mitogen (Sharon, 1983).

Marsupial studies that measure lymphocyte responses to mitogens have been undertaken using a variety of culture media, cell densities, incubation times and proliferation assay techniques. Notwithstanding these differences, mitogen-driven assessment of metatherian lymphocytes has consistently demonstrated their capacity to generate a cell-mediated immune response. Species in which mitogen-driven
lymphocytes proliferation has been reported include the quokka (Ashman et al., 1972), the tammar wallaby (Ashman et al., 1976), the North American opossum (Fox et al., 1976), the South American or grey, short-tailed opossum (Monodelphis domestica; Infante et al., 1991; Brozek et al., 1992), the koala (Wilkinson et al., 1992b), the brushtail possum (Moriarty, 1973; Baker et al., 1998), the Matschie’s tree kangaroo (Montali et al., 1998) and the red kangaroo (Montali et al., 1998). Whilst proliferation responses have been documented, mitogen levels for optimal responses are reportedly higher than those required in eutherian mammals (Prasad et al., 1971; Moriarty, 1973; Brozek et al., 1992) and at least in some species, these responses appear to be reduced when directly compared with other mammals (Montali et al., 1998).

1.5.6.2.4 Antigen-driven responses
Whilst cell-mediated responses using polyclonal activators have been commonly demonstrated in metatherian species, in vitro responses to antigens are less widely reported. After prior immunisation with the antigen under study, peripheral blood leukocytes of koalas do not proliferate in response to the soluble antigens, bovine serum albumin (BSA) and ovalbumin (Wilkinson et al., 1992a) but do respond to PPD after injection with BCG. When challenged with both bovine and avian PPD, brushtail possum lymphocytes achieved stimulation indices (SI), a measure of the ratio of stimulated cells to unstimulated cells, of greater than three and up to 23 (Buddle et al., 1994), results that are considered significant responses in eutherian mammals (Kristensen et al., 1982b). Antigen specific lymphocyte stimulation assays on both wild caught and captive Long-footed potoroos demonstrated no significant exposure to M. avium by the wild caught animals and significant levels of exposure to this pathogen by captive animals (Phelan, 1996).

1.5.7 Molecules of the Immune System
There are a number of effector molecules involved in the mediation of the immune response. Immunoglobulins and cytokines are produced as part of the specific immune response after appropriate activation by antigen. Other molecules, such as the acute phase proteins produced by the liver and the family of complement proteins found in serum, form part of the innate response to antigen and are produced immediately the antigen is detected.
1.5.7.1 Complement

In eutherians, the complement system of serum and cell surface proteins consists of approximately thirty components that assist in the execution of humoral immune responses and inflammation. During activation of the complement system, specific serum proteins or their cleavage products mediate a number of different immune functions. Amongst these are opsonisation, osmotic lysis of target cells, regulation of local inflammation and interaction with antibody molecules to clear immune complexes (Benjamini et al., 2000). Opsonisation, or coating of material with molecules that increases their ability to be phagocytosed, occurs via C3b deposition on the surface of leukocytes that possess receptors for this particular molecule. Cytolysis of target cells occurs as a result of the formation of a pore structure known as the membrane attack complex. Complement system cleavage products are largely responsible for the local inflammation response and complement proteins assist in the clearance of soluble immune complexes by binding to the constant region of antibody molecules (Abbas et al. 1997). Clearly, the complement system is an important component of the initial response to injury or infection, yet it has received little attention in the study of the marsupial immune response. Early studies of marsupial complement function were undertaken over thirty years ago (Wirtz and Westfall, 1967) on the North American opossum and more recently on the grey short-tailed opossum by Koppenheffer et al. (1998). Wirtz and Westfall (1967) surveyed the complement system of *D. virginiana* and found that opossum serum contained similar hemolytic units to that of guinea pig serum. Lynch et al. (1993a) demonstrated the presence of the third component of complement, C3, in the serum of the quokka and also demonstrated that, similar to eutherian mammals, quokkas possess both classical and alternative complement pathways. Koppenheffer et al. (1998) also confirmed that both the alternative and classical pathways exist in the complement system of *D. virginiana*.

1.5.7.2 Acute-phase proteins

Acute phase response (APR) proteins are produced in the liver in response to an infection or an injured tissue. They are produced by most members of the animal kingdom and are responsible for returning the physiological processes of the host to within normal levels (Baumann and Gauldie, 1994). When pathogens invade the host, APR proteins cause an increase in host body temperature, synthesis of a variety of hormones and promotion of the deposition of complement components onto microbial
surfaces making them targets for phagocytosis or direct lysis (Roitt, 1991). The only study of acute phase proteins in metatherians to date is by Richardson et al. (1998), who investigated the production and identity of these molecules in *M. domestica*. In response to injections with LPS, they found that while there were some differences in the regulation of albumin synthesis, serum proteins produced in response to this bacterial component were similar to those found in the eutherian acute-phase response.

### 1.5.7.3 Humoral Immune Responses

In eutherian mammals, B cells provide humoral or antibody-mediated immunity. The primary antibody response in a host occurs following initial exposure to the antigen where antigen specific memory cells are generated. Upon secondary challenge with this same antigen, memory B cells are activated and are thus able to mount an increased antibody response (Abbas *et al.*, 1997). In secondary immune responses, antibodies produced by memory B cells will bind the antigen forming an immune complex. These complexes are rapidly transported to areas of lymphoid aggregations in the secondary lymphoid tissue where they are phagocytosed by macrophages or dendritic cells (Szakal, 1989). This stimulates the formation of germinal centres that also contain antigen-presenting B cells. In the germinal centre, T cells are in intimate contact with B cells and promote the production of cytokines. After appropriate stimulation, B cells migrate to the medullary area in lymphoid tissue where they mature into plasma cells and produce the antigen specific antibody (Szakal, 1989).

Many of the early studies of the humoral immune response of marsupials dealt with responses to T-dependent antigens such as sheep red blood cells and bacteria. In eutherians, responses to these antigens require assistance from T helper cells for the development of antibody-producing plasma cells (Roitt, 1991). Results from these studies suggested that metatherian mammals were capable of mounting a quantifiable response to these antigens. As techniques improved and investigations of eutherian immunoglobulin classification continued, studies characterising the number and type of antibody classes present in metatherians were performed. Molecules with the physical properties of IgM and IgG were identified using traditional protein isolation techniques in the South American opossum (Rowlands and Dudley, 1969) the koala (Wilkinson *et al.*, 1991) and the North American opossum (Shearer *et al.*, 1995).
Four of the five major eutherian classes of immunoglobulin (IgG, IgM, IgA and IgE) were also identified in the macropod marsupial, the quokka (Stanley, 1983).

Renewed research interest coupled with advances in the techniques of molecular biology has allowed a more extensive investigation of the marsupial immunoglobulin system over the past decade. Studies of structural elements of these molecules have included identification of both heavy chain and light chain components (Aveskogh and Hellman, 1998, Belov et al., 1998; Miller et al., 1998; Lucero et al., 1998; Miller et al., 1999; Belov et al., 2001) and the cloning of the polymeric immunoglobulin receptor in the brushtail possum (Adamski and Demmer, 1999) and the tammar wallaby (Taylor et al., 2002).

Despite accounts of sluggish secondary responses in some marsupial immunisation studies (Wilkinson et al., 1992a), there have been successful reports of marsupial vaccination trials against a select number of disease-causing organisms. A vaccination trial against lumpy jaw, a bacterial disease that causes necrosis in the jaws of macropods, successfully prevented reinfection in tammar wallabies that demonstrated primary and secondary protective antibody responses similar to those mounted by eutherian mammals (Blanden et al., 1987). Tammar wallabies immunised with a *T. gondii* vaccine developed for use in sheep and goats, showed a variable response to immunisation with some animals contracting acute disease (Lynch et al., 1993b). However, animals that had seroconverted prior to this study were afforded protection to a debilitating disease course and survived deliberate re-infection. Sero-negative animals contracted severe disease or died. This lack of response to vaccination also occurs in some individuals within eutherian populations. However, the survival of the seropositive tammar wallabies after deliberate re-infection suggests that in general, this species is capable of mounting an effective protective memory response against this intracellular pathogen.

Brushtail possums vaccinated with live BCG (an attenuated *M. bovis* strain) were also afforded some protection from subsequent disease when later challenged with *M. bovis*. This protection was dependent of the route of vaccine delivery and was optimal when delivered at mucosal sites (Buddle et al., 1997).
1.5.7.4 Cytokines - Molecules that Regulate the Immune Response

Cytokines are cell-derived immunoregulatory proteins that are secreted by leukocytes and other nucleated cell types (Thomson, 1998). They play equally important roles in both the innate and specific branches of the immune response and are essential for immunobiological processes that include haematopoiesis, lymphoid organ development and modulation of the inflammatory response.

1.5.7.4.1 The role of cytokines in host defence processes

There are a number of cytokines that play pivotal roles in the mediation and regulation of innate immunity in eutherian mammals. These include, but are not limited to, the Type I Interferons (IFN), Tumour Necrosis factor alpha (TNF-α), Interleukin-1 (IL-1) and Interleukin-10 (IL-10) (Abbas et al., 1997). Of these molecules, TNF-α, IL-10 and the Type I IFNs, are also known to influence the type and duration of the specific immune response. The promotion or down-regulation of the immune response is an important factor in the control of response to disease, so it is essential to understand the mode of action of these regulatory molecules in captive marsupial species.

The identification of marsupial cytokines has been limited to molecular studies undertaken in the brushtail possum and the tammar wallaby, and two accounts from in vitro functional studies. A 15 to 17 kDa protein was isolated from lipopolysaccharide (LPS) stimulated opossum macrophages that was presumed to be Interleukin-1 based on a thymocyte co-stimulation assay (Brozek and Ley, 1991). Wilkinson et al. (1992b) reported an Interleukin-2-like molecule produced by mitogen stimulated lymphocytes of the koala, that was identified by its ability to sustain the viability of PHA stimulated lymphoblasts.

Molecular studies have been more fruitful in identifying the presence of gene sequences for cytokine molecules in metatherian mammals. The cDNA sequences of TNF-α in the possum (Wedlock et al., 1996) and the tammar wallaby (Harrison et al., 1999), lymphotoxin alpha and beta in the tammar wallaby (Harrison and Deane, 1999a and b), IL-10 and IL-1β in the possum (Wedlock et al., 1998 and 1999b) and IL-5 in the tammar wallaby (Hawken et al., 1999) have all been identified in the past decade. Recently, the immunoregulatory Type I Interferon family of genes has also
been found in the tammar wallaby (personal communication, Dr. G. A. Harrison, UWS).

**Type 1 Interferons**

The Type I interferons, IFN-α (leukocyte interferon) and IFN-β (fibroblast interferon), are multifunctional cytokines that play a role in both innate and specific immunity. In eutherian mammals, they are encoded by over 20 intronless genes and are produced in the response to invasion of host cells by viruses, bacteria and other antigenic stimuli (Kontsek and Konsekova, 1997). Type II interferon, Interferon-gamma (IFN-γ), is encoded by a single gene and is produced by effector T cells (Müller et al, 1994; Siegal et al, 1999).

Expression of Type I IFN molecules causes an increase in the expression of MHC Class 1 antigens and also enhances the activity of Natural Killer Cells (Tovey et al., 1996). Specific immunity is also strongly influenced by Type I IFNs since their presence directs the production of other cytokine molecules, particularly those associated with a Type 1 cytokine response - Interleukin 2, IFN-γ and TNF-α (Wattring et al., 1998; Benjamiini et al., 2000). Type I IFNs are expressed in many cells upon viral infection, which contrasts markedly with IFN-γ, which is predominantly produced upon antigen stimulation of T cells.

As part of their ability to modulate the immune response, Type I IFN molecules are able to inhibit cell growth and affect the development and differentiation of cell lines. In recent times, dendritic cells have been identified as the principal interferon-producing cell population in peripheral human blood (Siegal et al., 1999).

**Tumour Necrosis Factor Alpha (TNF-α)**

Cells of the monocyte/macrophage lineage, T cells and Natural Killer cells all produce TNF-α in vitro (Cockfield et al., 1993). This cytokine has many roles in immune responses ranging from stimulation and recruitment of lymphocytes through to the activation of macrophage antimicrobial responses. Regulation of the expression of TNF-α is essential for an effective immune response against intracellular pathogens, particularly mycobacterial species (Eriks and Emerson, 1997; Saunders and Cooper, 2000) and early downregulation of this predominantly macrophage-derived cytokine may allow intracellular bacteria to successfully establish inside
macrophages (Shiratsuchi et al., 1999). In mycobacterial infections, TNF-α contributes to the development of granulomas (Gan et al., 1995) and if produced at levels above those required, may cause damage to the surrounding tissue and subsequent immunopathology.

Interleukin 1 (IL-1)
IL-1 may be synthesised following bacterial challenge to the host or after exposure to cytokines such as TNF-α. It is produced by activated mononuclear phagocytes and endothelial and epithelial cells. Like TNF-α, one of the primary functions of IL-1 is to control the host inflammatory response (Abbas et al., 1997). In marsupials, IL-1 was first reported, but not confirmed, in a functional and electrophoretic study of the supernatants of LPS-treated macrophages from M. domestica (Brozek and Ley, 1991). Later, this molecule was characterised in the brushtail possum using molecular techniques by Wedlock et al. (1999b), who amplified the cytokine sequence from LPS-treated alveolar macrophages. The recombinant protein produced from this amplified product demonstrated functional bioactivity when tested in a possum thymocyte assay.

Interleukin 10 (IL-10)
Although originally known as cytokine synthesis inhibitory factor, IL-10 is better described as an immunomodulatory molecule since it suppresses and also up-regulates the production of other regulatory molecules. It is produced by a variety of cell types including T and B lymphocytes, monocytes, macrophages and neutrophils (Abbas et al., 1997). IL-10 is best known as a negative regulator of the immune response that suppresses the production of Type 1 cytokines such as Interleukin-2 (IL-2) and IFN-γ. Macrophage production of TNF-α and IL-1 is also down-regulated by this molecule which ultimately affects both innate and specific immune responses. Some examples of the specific immunoregulatory effects of IL-10 include down-regulation of macrophage MHC Class II expression, decreased macrophage nitric oxide production (Murray et al., 1997) and the inhibition of one-way mixed lymphocyte culture responses (Bejarno et al., 1992).

Counter-regulatory cytokines such as IL-10 are necessary to downregulate the production of inflammatory cytokines such as IFN-γ and TNF so that pathological damage due to overexpression of these molecules is avoided. Thus, they play a
significant role in infections where macrophages are the host cells for pathogens. The effects of high levels of IL-10 are dominant over the effects of other immunostimulatory molecules such as T cell produced IFN-γ (Schaible et al., 1999), which suggests that appropriate levels of this cytokine determine the ultimate effectiveness of Type I cytokines such as IL-2 and IFN-γ.

Immunoregulatory molecules clearly play a significant role in host defence and must be identified in order to investigate their role in disease susceptibility and progression. To date, IL-10 cDNA has been cloned and sequenced in only one marsupial species, the brushtail possum (Wedlock et al., 1998) where it was shown to have less than 60% sequence similarity to eutherian mammals.

1.5.8 Immune Response to Mycobacterial Disease

1.5.8.1 Granuloma Formation

Mycobacterial species proliferate within the cells of the host. In response to this incursion, cytokines produced during the co-operative activation of macrophages and T cells, stimulate fibroblast proliferation and the synthesis of collagen. A granuloma forms that ultimately leads to ‘wallowing off’ of the infection site (Abbas et al., 1997; Orme and Cooper, 1999). If successful, this response enables the host animal to control and confine the mycobacteria, thus limiting the aggressive effects of the disease. If unsuccessful, hypersensitivity reactions to the bacteria and its products may develop, that may result in the formation of structures such as caseous lesions, pulmonary cavitation and granulomatous skin responses.

Cytokines play a major role in the construction of an effective granuloma. TNF-α is required in this process but may cause tissue damage if overexpressed (Orme and Cooper, 1999; Schaible et al. 1999). The balance between IL-10 and IL-12 also contributes to granuloma development by regulation of the granulomatous response, which directly affects the ability of the host animal to contain the infection (Agostini et al., 1998).

1.5.8.2 Marsupial Immune Responses to Mycobacteria

The importance of effective granuloma formation in marsupials is apparent in animals where this response was not evident or where the response proved ineffective. Mycobacterial disease in brushtail possums has been more extensively studied than in
other metatherian species and reports of necrotic granulomas (Buddle et al., 1994) and disseminated disease (Jackson and Morris, 1996) are common.

Macrophages are the principal effector cells that control the progress of mycobacterial infections in eutherians (Carpenter et al., 1998). Thus, a difference in the roles of these cells or a deficit in their functioning may contribute to the susceptibility of marsupials to mycobacterial infection. Histochemical staining of these cells in possum monocytes indicates that these cells are deficient in enzymatic activity (Barbour, 1972). Another property of the macrophage that may contribute to granuloma formation is the ability of these cells to respond to chemotactic factors released at the site of infection. This attribute may not be present in metatherian species, since Moriarty and Thomas (1986) demonstrated the lack of responsiveness of possum peritoneal macrophages to treatment with agents known to be chemotactic for guinea pig cells.

Since resistance to tuberculosis is a function of cell mediated immunity (Collins and Campbell, 1981) the susceptibility of marsupials to mycobacterial infection suggests a defect in this aspect of their immune response. However, in the brushtail possum, this defect is not related to a failure to produce specific acquired cellular immune responses to mycobacterial antigens. Studies have shown that possums infected with M. bovis or immunised with M. bovis BCG mount strong peripheral blood lymphocyte (PBL) blastogenic responses to bovine purified protein derivative (PPD) (Buddle et al., 1994). Koalas and potoroos also recognise mycobacterial antigens in vitro (see earlier discussion). Notwithstanding results of this work, reports of lowered or deficient cell-mediated immunity in both healthy and disease-affected marsupials have been limited but consistent (Wilkinson et al., 1992a; Montali et al., 1998; Stone et al., 1998).

In order to more clearly define the defence strategies available to marsupials for a successful immune assault on intracellular pathogens, there is a requirement to clarify the ability of metatherians to effect these responses. To do this, benchmark responses for marsupials need to be documented, using the information currently available regarding the mechanism of eutherian immune responses to intracellular pathogens. The first line of defence that any bacterial pathogen encounters is the neutrophil. In humans, neutrophils can kill M. tuberculosis (Brown et al., 1987) and M. avium-M.
intracellulare using both oxidative and non-oxidative defences (Ogata et al., 1992). The importance of a functioning granulocyte oxidative response is reinforced by studies that suggest that human subjects with Chronic Granulomatous Disease (CGD), an inherited disorder of phagocyte dysfunction which is manifested by the poor or absent production of reactive oxygen intermediates, are susceptible to M. avium (Ohga et al., 1997). In chronic mycobacterial infections, the continuous production of NO is also thought to play a role in suppression of this bacterium (Schaible et al., 1999).

If the microorganism successfully bypasses the innate immune mechanisms of the host, the immune response to intracellular bacterium is primarily mediated by T lymphocytes and macrophages (Doherty and Sher, 1997). Since the organisms are able to survive inside macrophages, they are protected from attack by antibodies and the complement proteins (Splinter et al., 1996). Thus, it is the immunoregulatory molecules that then become responsible for the isolation and eradication of the pathogen. Increases in TNF-α production by mononuclear cells activates the antimicrobial properties of macrophages and promotes a granulomatous response (Schaible et al., 1999). Counter regulatory cytokine behaviour contributes to the outcome of the response and the pro-inflammatory molecule, IL-1β, and the immunosuppressive molecule, IL-10, may both play a significant role in the immunopathology associated with the immune response.

The eutherian immune response is clearly complex and relies on the interplay between a number of different elements. Therefore, before a definitive explanation for the apparent susceptibility of marsupials to intracellular pathogens can be provided, information regarding the fundamental immunological strategies available to these animals must first be obtained.
1.6 Project Rationale and Aims
The immunological response to invading pathogens is rarely resolved by a single event. It is generally a complex interplay of interconnected responses that are determined by the nature of the pathogen and the immunocompetence of the host. Both cellular and molecular components play pivotal roles in the response to disease, therefore in any investigation of immune function, there is a need to examine the capacity of both the innate and adaptive branches of the immune system. Intracellular pathogens, which by their very nature evade direct humoral responses, require a combination of both the non-specific and cell-mediated specific responses of a functioning immune system to effect their destruction or effective isolation. These aspects of the marsupial immune response were investigated by firstly identifying and then analysing the in vitro immunological responses of immune cells and the molecules they produce with particular reference to T cell mediated responses and innate cellular defences.

The *Macropodinae* marsupial, the tammar wallaby (*M. eugenii*) was chosen as a model species for this study. Using this animal, benchmark cellular immune responses were established for macropod marsupials. This facilitated the study of the immune responses of another member of the *Macropodinae*, the Rufous Hare-wallaby (*Lagorchestes hirsutus*) and two members of the *Potoroinae*, the Long-nosed potoroo (*Potorous tridactylus*) and the Long-footed potoroo (*Potorous longipes*).

1.6.1 Hypothesis
As adults, eutherian and metatherian mammals share similar anatomical and physiological attributes. Therefore, it is reasonable to assume that the functionality of marsupial immune systems is also similar to their eutherian relatives. However, to date, reports of “retarded” and “primitive” metatherian immune responses have led to the suggestion that differences exist between eutherian and marsupial responses to antigen (Wilkinson *et al.*, 1994; Jurd, 1994). As yet, these assumptions remain largely unchallenged due to the lack of comprehensive studies of basic immunological systems in metatherian mammals. The current study was initiated in response to the lack of fundamental knowledge describing the basic immune responses of marsupials, particularly within the family *Macropodidae*. The hypothesis investigated during this study focussed primarily on the contrary assumption that ‘marsupials possess cellular immune capacity similar to their eutherian relatives’.
1.6.2 Project Aims

Since the scope of this project was to investigate the capacity of metatherian immune cells to mount a broad range of immunological responses, the study was segregated into a number of discrete aims:

1. Isolation, identification and characterisation of marsupial immune cells and their products.

2. Development and optimisation of immunological protocols to measure marsupial immune responses.


4. Evaluation of the in vitro cellular immune competence of endangered macropodid animals by application of tests optimised in the model animal species.

5. Investigation of the cellular and immunopathological affects of mycobacterial disease on opportunistic samples of the endangered macropods, the Long footed potoroo and the Rufous Hare-wallaby.
CHAPTER TWO

Materials and Methods

2.1 Introduction

The investigation of immune function requires the development and optimisation of procedures that first isolate and then identify the cells that are responsible for the generation of the particular immune response. There are currently few specific reagents or experimental protocols that can be used for these assessments in marsupials, with the availability of reagents for marsupial immunological research restricted to a number of antibodies raised to immunoglobulin molecules by individual research groups, and to a limited number of species cross-reactive antibodies that were developed from evolutionarily conserved sequences of human cell-surface markers (Jones et al., 1993). Similarly, the protocols used to assess in vitro immune function are restricted to those developed and tested on eutherian species rather than in specific marsupial models. Since the work undertaken for this thesis was concerned with the assessment of both innate and cell-mediated immune responses in marsupials, one of the principal study objectives was the establishment and modification of protocols that could achieve this aim. This involved the development of new protocols, together with the customisation and validation of existing immunological and biochemical methods currently used to investigate eutherian immune cell function.

This chapter describes the methods employed to identify, enumerate and characterise the immunological function of the cells and tissues of the tammar wallaby and three other small wallaby species, the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo.

2.2 Materials

2.2.1 Reagents and Consumables

All plasticware used for sample preparation and processing was manufactured from polypropylene (unless otherwise stated) to avoid non-specific cell activation. Where appropriate, reagents for tissue culture experiments were purchased endotoxin-free and tissue culture tested. All reagents, including tissue culture media, were filter sterilised through 0.22µm Nalgene filters (Pall Gelman Laboratory; Michigan, USA)
immediately before use. Greiner Tissue culture grade culture vessels and microtitre plates, 10mL and 50mL centrifuge tubes, 5mL culture tubes and gamma-irradiated filter tips (used for tissue culture and molecular analyses), were all purchased from Crown Scientific (Moorebank, Australia). Plastic disposable petri dishes were obtained from Bacto Laboratories (NSW, Australia) and bacteriological agar from Oxoid Ltd. (Hampshire, England). With the exception of oligonucleotide primers, reagents and enzymes used for molecular biology protocols were purchased from Promega (NSW, Australia) unless otherwise stated. Primers were obtained from GeneWorks Pty Ltd (Adelaide, Australia), Sigma Genosys (Missouri, USA) and Beckman (NSW, Australia). Coomassie® Plus Protein Assay Reagent was purchased from Pierce (Illinois, USA). All other materials used in this study were purchased from Sigma (Missouri, USA).

2.2.2 Media

Cell isolation and proliferation experiments were performed in both serum-supplemented and serum-free media. Serum-enriched media included Roswell Park Memorial Institute (RPMI)-1640 media containing 2mM glutamine and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (RPMI) supplemented with either pooled tammar wallaby serum (TWS) or foetal bovine serum (FBS) at levels of 5 and 10%. Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS was also used for one series of experiments.

Tammar wallaby serum was collected from animals within the test colony (2.2.3.1). Both standard foetal calf serum (FCS) and a low-haemoglobin FBS were used for comparative experiments. All sera were heat-inactivated at 56°C for 45mins and filter sterilised before use.

Three different serum free media were also used in this study. These included RPMI-1640 containing 2mM glutamine and HEPES buffer without serum supplementation, Quality Biologicals serum-free media (QBSF)®-51, a commercially prepared, low protein (<45μg/mL) serum-free media containing BSA, transferrin and insulin at proprietary levels, and Hybrimax, a cholesterol-containing media that also contained these three supplements.
Hanks Balanced Salt Solution without phenol red (HBSS), HBSS without phenol red, calcium and magnesium ions (HBSS-), and Phosphate Buffered Saline (PBS) were used to wash cell preparations or as suspension media as described.

2.2.3 Stock Solutions

A variety of stock solutions were used in many of the in vitro analysis undertaken in this study. The most commonly used reagents and their methods of preparation are described below.

Stock solutions of mitogens phytohaemagglutinin (PHA), Concanavalin A (Con A), Pokeweed Mitogen (PWM), Lipopolysaccharide (LPS), cell stimulating agents phorbyl myristate acetate (PMA) and Ionomycin, the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), antibiotics and MTT were dissolved separately in buffered salt solutions or media to stock concentrations (see Table 2.1), aliquoted into small working volumes and stored frozen at -20°C until required with the exception of fMLP stock preparations which were prepared in dimethylsulfoxide (DMSO) and stored at -70°C. Apart from the MTT solution that was used directly at stock concentration, all other stock solutions were thawed on ice and diluted to working concentrations immediately before use. Stock concentrations and the working concentrations prepared from them are shown in Table 2.1.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Stock concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT</td>
<td>5mg/mL in media</td>
<td>500μg/mL</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>10000Units/mL/10mg/mL</td>
<td>100Units/100μg/mL</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>250μg/mL</td>
<td>250μg/mL</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10mg/mL</td>
<td>100μg/mL</td>
</tr>
<tr>
<td>PHA</td>
<td>10mg/mL</td>
<td>200μg/mL</td>
</tr>
<tr>
<td>Con A</td>
<td>5mg/mL</td>
<td>100μg/mL</td>
</tr>
<tr>
<td>PWM</td>
<td>3.34mg/mL</td>
<td>100μg/mL</td>
</tr>
<tr>
<td>LPS</td>
<td>10mg/mL, 1mg/mL</td>
<td>100μg/mL</td>
</tr>
<tr>
<td>PMA</td>
<td>10μg/mL</td>
<td>1ng/mL, 10ng/mL, 1μg/mL</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>10μg/mL</td>
<td>1ng/mL, 10ng/mL, 1μg/mL</td>
</tr>
<tr>
<td>fMLP</td>
<td>10⁻³ M in DMSO</td>
<td>10⁻⁵ – 10⁻⁸ M</td>
</tr>
</tbody>
</table>

Table 2.1: Stock Solutions and Working Concentrations used in in vitro Cellular Assays
Serum-opsonised zymosan (SOZ) was prepared by suspension of 10mg of zymosan in 1mL of PBS, followed by the addition of an equal volume of freshly isolated serum. This solution was incubated for 30mins at 37°C and used at dilutions of 1/10 and 1/20 in experimental assays as required. When zymosan-activated serum was required, the serum solution was removed from the above preparation by centrifugation at 500g for 10mins.

*Escherichia coli*-activated serum and *Staphylococcus aureus*-activated serum were prepared in a similar manner with the exception that $10^8$ cells/mL of bacteria were incubated with serum in place of zymosan. In the measurement of chemotactic properties, nutrient broth and supernatants of *E. coli* and *S. aureus* activated nutrient broth were tested for their ability to promote cell movement. Bacterial-activated nutrient broths were prepared by overnight growth of the bacterial inoculum in 10mL of nutrient broth, followed by centrifugation at 500g for 10mins to pellet bacterial cells. Supernatants of these cultures were recovered for use as test agents in polarisation, chemotaxis and oxidative burst studies. Bacteria were recovered by centrifugation and suspended in 1mL of PBS for use in phagocytic assays.

Commercially purchased 1.1µm and 3µm latex beads were also used in phagocytosis experiments after suspension in HBSS- at $10^6$ beads/mL. In some experiments, bead suspensions were opsonised with an equal volume fresh serum at 37°C for 30mins.

### 2.2.4 Animals
The animals studied in this work are all members of the family *Macropodidae*. The tammar wallaby and Rufous Hare-wallaby are classified within the sub-family *Macropodinae* and the Long-footed and Long-nosed potoroos are in the sub-family *Potorinae*. Also included in this study were single opportunistic samples obtained from another potoroine, the Brush-tailed bettong, and two members of the *Monotremata*, the short-beaked echidna and the platypus.

#### 2.2.4.1 Tammar Wallaby (*Macropus eugenii*)
The tammar wallaby is one of the smallest species of the genus *Macropus*, weighing between 4 and 10kg. They are found living in areas that support shrub habitats that include dry sclerophyll forests, scrubs and heathlands (Cronin, 1991). In captivity,
low dense shrubs and covered areas must be provided to meet their need for shelter and shade.

The tammar wallaby is one of only two macropodid species that shows a seasonal pattern of breeding. The breeding season occurs after the summer solstice, from January to July and most young are born in late January and throughout February and March (Renfree, 1981b). A single birth is immediately followed by post-partum mating. The embryo that forms as a result of this mating remains dormant during lactation and typically does not reactivate until after the summer solstice or when the suckling stimulus of the pouch young (PY), born in the previous season, is removed (Tyndale-Biscoe and Janssens, 1988). Female wallabies achieve sexual maturity while still suckling at approximately 8 months of age, males much later at approximately 2 years old (Cronin, 1991).

The gestation time for *M. eugenii* is approximately 26-30 days, the oestrus cycle is one day longer (Hinds et al., 1990). After birth, the young tammar remains attached to the teat until it reaches 105 days old. It continues to suckle and, during this time, physiological processes continue to develop until it is able to maintain its own body temperature. The young animal is then able to leave the pouch permanently at approximately 250-270 days. Suckling continues until lactation has ceased, usually by an age of 43 weeks.

Tammar wallabies may live for up to 14 years. It is not unusual for the first born pouch young to be lost and mortality is also high in juvenile tammar wallabies during their first summer (Cronin, 1991; Strahan, 1998). At the onset of cold, wet weather, many animals die if conditions during the summer season have been harsh.

*M. eugenii* were once widely distributed across the continent but are now limited to island populations. These include Kangaroo Island in South Australia, where they are found in large numbers, and a number of islands off the Western Australian coast including Garden Island, near Fremantle. A small, isolated population of tammars thought to be the only remaining mainland population of this species still exists in southwest Western Australia. In general, tammar wallaby populations are vulnerable due to their isolation and continued threats presented by changes in habitat due to
scrub clearing, fire regimes and the predation behaviour of introduced species (Hinds et al., 1990; Cronin, 1991; Maxwell et al., 1996; Strahan, 1998; Mate et al., 1999).

The tammar wallabies used in this work are founded from a colony of animals that originated in Kangaroo Island (South Australia). These animals were members of a captive-breeding colony housed at Macquarie University Fauna Park (NSW, Australia). Both male and female animals formed part of the study group, which originally contained one breeding male and 11 females. Animals designated for this study were placed into a separate yard where they were allowed to breed. Most young resulting from these matings were transferred to other yards once they were independent. Some juveniles remained in the group to replace animals that died during the course of the study.

The experiments described herein were carried out over a three and a half year period that included three breeding seasons with sampling continuous throughout the 12 months of the year. The length of the study and the uninterrupted breeding cycles resulted in the inclusion of samples from female animals during periods of pregnancy, lactation, weaning and throughout normal oestral cycles.

During the course of the study, between late October, 1998 through to December 1998, the tammar wallaby test group, along with the remainder of the animals held at Macquarie University Fauna Park, were inadvertently exposed to an infectious agent that caused the death of approximately 30% of the colony. The agent was suspected to be an orbivirus (personal communication Dr. Karrie Rose; Taronga Zoo, Sydney) that may have been the primary cause of death or served to reduce the fitness of animals so that they succumbed to disease caused by a protozoan parasite T. gondii. At the time of writing, the causative agent of this outbreak has not yet been clarified and is still under investigation.

In total, blood and/or tissues were obtained from 44 different tammar wallabies during this study. Details of their history are shown in Table 2.2. Some animals were tested in the early phase of the study for the presence of serum antibodies to T. gondii as part of a separate research project (personal communication, Prof. Desmond Cooper, Macquarie University). Seronegative and seropositive animals are also indicated in Table 2.2. Animal samples were obtained with the consent of Macquarie
University Animal Care and Ethics Committee under Animal Research Authority Number 97042. Opportunistic blood and tissue samples were also made available by Dr. Genevieve Magarey and Dr. Karen Mate of the CRC for the Conservation and Management of Marsupials (Macquarie University).
<table>
<thead>
<tr>
<th>Animal</th>
<th>Gender</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>1008*</td>
<td>♀</td>
<td>1 offspring during study. Deceased.</td>
</tr>
<tr>
<td>1068</td>
<td></td>
<td>Opportunistic sample – not from main study group.</td>
</tr>
<tr>
<td>12F3CODT</td>
<td>♀</td>
<td>Mother of 1961. 4 offspring over study period.</td>
</tr>
<tr>
<td>12F5O94T</td>
<td></td>
<td>1 offspring before demise. Deceased.</td>
</tr>
<tr>
<td>131</td>
<td>♂</td>
<td>Opportunistic sample – not from main study group.</td>
</tr>
<tr>
<td>1331</td>
<td></td>
<td>Opportunistic sample – not from main study group.</td>
</tr>
<tr>
<td>1338B8ST</td>
<td>U</td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1334F5DT</td>
<td>U</td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1334F94T**</td>
<td>♂</td>
<td>Juvenile male.</td>
</tr>
<tr>
<td>1336</td>
<td>♀</td>
<td>Opportunistic sample – not from study group.</td>
</tr>
<tr>
<td>13367E8T*</td>
<td>♀</td>
<td>Opportunistic sample – not from study group.</td>
</tr>
<tr>
<td>133DOC7T</td>
<td></td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1378B8AT</td>
<td>♀</td>
<td>3 offspring during study.</td>
</tr>
<tr>
<td>1400</td>
<td>♂</td>
<td>Breeding male.</td>
</tr>
<tr>
<td>1503</td>
<td>♀</td>
<td>Offspring of 1008. Deceased.</td>
</tr>
<tr>
<td>1515</td>
<td>♀</td>
<td>Juvenile female.</td>
</tr>
<tr>
<td>1526*</td>
<td>♀</td>
<td>Died on capture. At necropsy possessed enlarged nodes and pale spleen</td>
</tr>
<tr>
<td>1538</td>
<td>♀</td>
<td>Opportunistic sample. Previously received FSH/LH injections. Enlarged nodes at necropsy.</td>
</tr>
<tr>
<td>1580</td>
<td>♂</td>
<td>Juvenile male, offspring of 1612. Transferred out of yard.</td>
</tr>
<tr>
<td>1583</td>
<td>♀</td>
<td>Juvenile male.</td>
</tr>
<tr>
<td>1612**</td>
<td>♀</td>
<td>Mother of 1580. 3 offspring during study period.</td>
</tr>
<tr>
<td>1652**</td>
<td>♀</td>
<td>Mother of 1515. 3 offspring during study period. Died 31/10/00. Cause unknown.</td>
</tr>
<tr>
<td>1654</td>
<td>♀</td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1657**</td>
<td>♀</td>
<td>3 offspring during study period.</td>
</tr>
<tr>
<td>1817</td>
<td>U</td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1844*</td>
<td>♀</td>
<td>Mother of 1958.</td>
</tr>
<tr>
<td>1935</td>
<td>♀</td>
<td>Juvenile male. Deceased.</td>
</tr>
<tr>
<td>1957</td>
<td>♀</td>
<td>Offspring of 1844. Euthanased.</td>
</tr>
<tr>
<td>1960</td>
<td>♀</td>
<td>Juvenile female.</td>
</tr>
<tr>
<td>1961</td>
<td>♀</td>
<td>Juvenile male. Transferred out of yard.</td>
</tr>
<tr>
<td>1994</td>
<td>♀</td>
<td>Offspring of 8021. 1 offspring during study period.</td>
</tr>
<tr>
<td>1DA6338T</td>
<td>U</td>
<td>One-off sample.</td>
</tr>
<tr>
<td>1E722CFT</td>
<td></td>
<td>No offspring during three year study period.</td>
</tr>
<tr>
<td>1EF4CADT</td>
<td>♀</td>
<td>Juvenile male.</td>
</tr>
<tr>
<td>1EF4CB2T</td>
<td>♀</td>
<td>3 offspring during study period.</td>
</tr>
<tr>
<td>202**</td>
<td>♀</td>
<td>Deceased.</td>
</tr>
<tr>
<td>209*</td>
<td>♀</td>
<td>Euthanased when losing condition. Infected with coccidia (pers. comm. C. Bernard).</td>
</tr>
<tr>
<td>592</td>
<td></td>
<td>Opportunistic sample – not from study group.</td>
</tr>
<tr>
<td>6450*</td>
<td>♀</td>
<td>2 offspring over study period. Injured eye May '98. Deceased.</td>
</tr>
<tr>
<td>6693*</td>
<td>♀</td>
<td>2 offspring before demise. Deceased. At necropsy, lumpy jaw &amp; pulmonary congestion evident. Liver diffusely mottled with miliary white foci through the parenchyma.</td>
</tr>
<tr>
<td>8021*</td>
<td>♀</td>
<td>Mother of 1957. 3 offspring over study period.</td>
</tr>
<tr>
<td>8435</td>
<td>♀</td>
<td>Opportunistic sample – not from study group.</td>
</tr>
</tbody>
</table>

* T. gondii seronegative; ** T. gondii seropositive; FSH/LH Follicle Stimulating Hormone/Luteinising Hormone; U Unknown; Deceased = animals deceased in 98/99 summer season due to putative orbiviral infection.

Table 2.2: Tammar Wallaby Study Subjects
2.2.4.2 Potoroos

Animals in the *Macropodidae* subfamily *Potoroinae* are amongst the smallest of the macropodids and include animals from the *Potorous*, *Aepyprymnus* and *Bettongia* genera. The common Long-nosed potoroo (*Potorous tridactylus*) and the endangered Long-footed potoroo (*Potorous longipes*) were both subjects in this study and are described below.

2.2.4.2.1 Long-nosed Potoroo (*Potorous tridactylus*)

Long-nosed potoroos are fragmented in their distribution but are nonetheless classified as common (Cronin, 1991). These small macropods usually weigh between 0.66kg-2.07kg and are predominantly nocturnal animals that feed on fungi, insects, roots and seeds. They reach sexual maturaty at 12 months and are able to breed throughout the year with peaks in summer and late winter. Thirty-eight days after mating, a single young is born that leaves the pouch at approximately 15 weeks and suckles at foot for a further 5-6 weeks. Long-nosed potoroos can live to 12 years in the wild and are known to live at least this long in captivity.

2.2.4.2.2 Long-footed Potoroo (*Potorous longipes*)

Unlike the common Long-nosed potoroo, the Long-footed potoroo is a rare animal that was not described until 1980. This species is larger than the closely related Long-nosed potoroo and can be distinguished, as the name suggests, by its longer hindfeet. *Potorous longipes* is classified as endangered (Saxon *et al.* 1994) and the decrease in population numbers is thought to be a result of logging practices, fox predation and possibly disease (Maxwell *et al.*, 1996; Luikart *et al.*, 1997).

The Long-footed and Long-nosed potoroo species that were investigated during this study were housed at Healesville Animal Sanctuary (Melbourne, Australia) and were either originally wild caught or born in captivity (Table 2.3). The Long-footed potoroo colony was established in 1980 from four animals (Phelan, 1996). After early successes with captive breeding, the number of animals grew to 19. This period was followed by a reduction in breeding success that was associated with the presence of mycobacterial disease within the colony. This resulted in the colony number dwindling to one at the time of writing. At the commencement of the present study, the Long-footed potoroo species was thought to be more susceptible to mycobacterial disease than their Long-nosed relatives and for the purposes of this study, *P. tridactylus* was to serve as the control species. However, during the investigation, it became evident that both *Potorous* species were affected by this intracellular pathogen.
<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. longipes</em></td>
<td>224♀</td>
<td>Wild caught animal.</td>
</tr>
<tr>
<td></td>
<td>224PY**</td>
<td>Offspring of wild-caught animal. Pouch young, age unknown.</td>
</tr>
<tr>
<td></td>
<td>820054♀</td>
<td>Euthanased due to suspected mycobacterial disease. Positive to <em>M. avium</em> in LSA test (1996*).</td>
</tr>
<tr>
<td></td>
<td>990497</td>
<td>Offspring of 880195</td>
</tr>
<tr>
<td><em>P. tridactylus</em></td>
<td>860077♂</td>
<td>Euthanased due to ill-health. Old animal.</td>
</tr>
<tr>
<td></td>
<td>860257</td>
<td></td>
</tr>
<tr>
<td></td>
<td>980239♂</td>
<td>Euthanased with damaged spine. Skin lump at base of tail. Previous lesion compatible with mycobacteria.</td>
</tr>
<tr>
<td></td>
<td>990433♂</td>
<td>Euthanased with suspected mycobacterial infection. Originally from Melbourne Zoo. Father diagnosed with mycobacterioses.</td>
</tr>
</tbody>
</table>

**Table 2.3: Potoroo Study Subjects.** LSA Lymphocyte Stimulation Assay. *Results by G. Browning, reported in Phelan, 1996. **PY Pouch Young
2.2.4.3 Rufous Hare-wallaby (Mala) (Lagorchestes hirsutus)

The Rufous Hare-wallaby or Mala is one of four species of hare-wallaby from the genus *Lagorchestes* (subfamily *Macropodinae*) (Maxwell *et al*., 1996; Kirsch *et al*., 1997). They are amongst the smallest members of the macropod family weighing less than 2kg. Like most macropods, they are largely herbivorous in their dietary habits and prefer an environment of low shrubs to provide adequate shelter and shade (Johnson and Burbidge, 1998). Provided conditions are suitable, females reach sexual maturity at approximately 5 months of age, after which reproduction is continuous throughout the year (Cronin, 1991).

Rufous Hare-wallabies were once abundant in the spinifex areas of Australia but because of the predatory impact of introduced species and changes in fire regimes that altered vegetation patterns (Bolton and Latz, 1978), *L. hirsutus* is now critically endangered. The reduction of animal numbers on the mainland was so dramatic that in 1996 this species was classified as ‘extinct in the wild’ (Maxwell *et al*., 1996). Island populations exist off the Western Australian Coast on Bernier and Dorre Islands, but at the time of this study, mainland numbers were limited to a small managed colony of animals in the Tanami Desert, Northern Territory, held within an area of approximately 50 square kilometers.

A productive captive-breeding program in Alice Springs, with animals originally sourced from The Tanami Desert, has provided animals for both translocation and re-introduction programs (Johnson, 1999). The animals sampled in this study originated from this group (Table 2.4). The original Tanami source area was destroyed by wildfire in 1991, but after a period of successful breeding in captivity, Mala were reintroduced to the Desert at two different locations. Unfortunately, in both trials, predation by cats (*Felis catus*) caused high Mala fatalities (Gibson *et al*., 1994). More recently, the translocation of animals to Trimouille Island, off the northwest coast of Western Australia, has been more successful (Langford and Burbidge, 2001). On this predator-free island, Mala are successfully breeding and appear to be establishing a viable population.
<table>
<thead>
<tr>
<th>Number</th>
<th>Gender</th>
<th>Age</th>
<th>Animal Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>241</td>
<td>♀</td>
<td>8yrs</td>
<td>Euthanased with suspected mycobacterial disease.</td>
</tr>
<tr>
<td>439</td>
<td>♂</td>
<td>5yrs</td>
<td>Euthanased due to ill-health.</td>
</tr>
<tr>
<td>89518722</td>
<td>♂ ♂</td>
<td>5yrs</td>
<td>Deceased. Trauma victim.</td>
</tr>
<tr>
<td>960106</td>
<td>♀ ♂</td>
<td>8.5yrs</td>
<td>Management euthanasia. Mild gut pathology evident at necropsy. No visible parasites.</td>
</tr>
<tr>
<td>960107</td>
<td>♂ ♂</td>
<td>6yrs</td>
<td>Management euthanasia. No visible parasites.</td>
</tr>
<tr>
<td>970500</td>
<td>♀</td>
<td>8yrs</td>
<td>Management euthanasia. No gross lesions. Cestodes present in small intestine.</td>
</tr>
<tr>
<td>970510</td>
<td>♀</td>
<td>7yrs</td>
<td>Management euthanasia. Parasites visible.</td>
</tr>
<tr>
<td>970518</td>
<td>♂</td>
<td>7yrs</td>
<td>Trauma victim. Swelling in left ear - aural haematoma. Cestodes visible and 2 x tape worms in gut.</td>
</tr>
<tr>
<td>970520</td>
<td>♂</td>
<td>7yrs</td>
<td>Management euthanasia. Nematodes in stomach and oesophagus.</td>
</tr>
<tr>
<td>990107</td>
<td>♂</td>
<td>1yr</td>
<td>Management euthanasia. Cestodes visible in small intestine.</td>
</tr>
<tr>
<td>990068</td>
<td>U U</td>
<td>U</td>
<td>Death due to capture myopathy.</td>
</tr>
</tbody>
</table>

U Unknown

**Table 2.4: Rufous Hare-Wallaby Study Subjects.** Information in this table was abstracted from autopsy reports (Alice Springs Desert Park Veterinary Clinic) prepared at the time of euthanasia.
2.2.4.4 Brush-tailed Bettong (Bettongia penicillata)

*Bettongia penicillata* is a small potoroid species that weighs between 1.1-1.6kg. Like *P. longipes* and *P. tridactylus*, it is a nocturnal animal and also has a diet that includes underground fungi, insects and seeds. The Brush-tailed bettong is sexually mature at approximately 6 months of age and is able to breed throughout the year, when a post-birth mating results in a viable embryo that is dormant until the pouch no longer supports the preceding young (Cronin, 1991). Bettongs were known to inhabit dry sclerophyll forests before the reduction in population numbers saw them classified as 'extinct in the wild' (Maxwell et al., 1996). An opportunistic blood sample was obtained from one of a small number of these animals that are kept in captivity at the Northern Territory Desert Park.

2.2.4.5 Monotremes

The only living members of the mammalian subclass Prototheria, the platypus and echidnas are distinguished from marsupial and eutherian mammals by their ability to lay soft-shelled eggs and their unique method of feeding their young through abdominal ducts rather than through formed teats (Strahan, 1998). During the course of this study, opportunistic blood samples from two members of the *Monotremata*, the Short-beaked echidna (*Tachyglossus aculeatus*) and the Platypus (*Ornithorhynchus anatinus*), were obtained from Taronga Zoo, Sydney.
2.3 Methods

2.3.1 Sample Collection

All blood and tissue samples from tammar wallabies were obtained from animals that appeared clinically normally at the time of sampling unless otherwise indicated. Samples obtained from other species were generally taken by veterinary staff during routine examinations or were obtained after euthanasia. Most animal samples from the Rufous Hare-wallaby and potoroos were obtained from animals suspected to be affected by disease. A number of Rufous Hare-wallaby samples were opportunistically obtained from clinically normal animals in the course of an animal management program.

2.3.1.1 Blood

A. Tammar wallabies

Blood sample collection occurred in the early mornings in order to minimise stress to the animals arising from capture and handling. Animals were caught using large custom-made nets, removed by the tail by trained animal handlers and held in hessian bags until the sample was obtained. Blood samples were taken from the lateral tail vein of the animals after swabbing the sampling site with 70% ethanol. 21 or 23 gauge needles attached to 5 or 10mL disposable syringes were used for all blood collections. Syringe contents were immediately transferred to potassium ethylenediaminetetra-acetic acid (EDTA) or heparin-treated blood collection vials (Becton Dickinson; New Jersey) and immediately inverted to avoid clotting. Blood for serum isolation was collected in an identical fashion but was added to polypropylene tubes without anticoagulant.

B. Other Species

As indicated previously, for all species other than the tammar wallaby, blood was collected by Veterinary Staff at the various sampling sites. These samples were placed into 3mL EDTA/heparin-coated tubes and shipped to the laboratory at room temperature (RT) or in insulated cooler packs.

2.3.1.2 Serum Isolation

Serum was recovered from collection tubes by standing at RT for 2 hours before ringing the formed clot with a wooden applicator. Blood was then refrigerated
overnight (4°C) to encourage clot retraction. Serum was isolated by centrifugation at 2000rpm for 10-20mins at 4°C. All serum used for cell studies was presterilised using a 0.22μm filter. Serum destined for cell culture and proliferation studies was subsequently heat inactivated at 56°C for 45mins.

2.3.1.3 Antibiotics
The antibiotics routinely used in this study included penicillin, streptomycin, gentamycin and the anti-fungal agent, amphotericin B. All wash solutions and media contained antibiotics unless otherwise specified. Endotoxin-free, filter sterilised, cell culture tested antibiotics were purchased as stock solutions in the concentrations stated in Table 2.1. Antibiotics were routinely added to a final concentration of 100U/mL penicillin, 100μg/mL streptomycin, 100μg/mL gentamycin and 25μg/mL amphotericin B unless otherwise stated. In warmer weather, antibiotics were added directly to whole blood samples during the preparation and set-up times to inhibit growth of any contaminating organisms.

2.3.1.4 Tissues
For tammar wallaby samples, animals were euthanased with a lethal injection of lethal barbitone (Virbac; Arnold of Reading, Victoria). For Mala and potoroo samples, animals were euthanased according to the site protocols approved by each institution’s animal care and ethics committee. In all cases, tissues were collected as quickly as possible after the animals’ demise. Potoroo tissue samples were obtained under Victorian Zoological Parks and Gardens Board ethics committee approval number 98012.

For cell culture work, organ sections were placed in HBSS-containing amphotericin and penicillin/streptomycin at double strength concentrations and transported to the laboratory in a cooled, insulated container.

For molecular work, tissue samples were placed into dry ice immediately after collection. All tissue samples were transported to the laboratory on dry ice and were stored at −80°C until processed.
For histological and immunohistological analyses, samples were placed immediately into 10% neutral buffered formalin or Bouin’s fixative and transported to the laboratory at RT. Tissues were transferred into ethanol, routinely processed and embedded in paraffin wax (Bancroft and Stevens, 1990).

2.3.2 Statistical Analyses
Statistical analyses were performed using Microsoft® Excel 97 Data Analyses software (Microsoft Corporation, USA). Wherever sample size permitted, analyses were performed in triplicate or replicates of five and results are presented as the mean ± one standard deviation unless otherwise specified. Where only duplicate results were obtained due to limited sample material, results are presented as the mean ± range/2 to demonstrate the presence or absence of noticeable trends. Where applicable, Student’s t-tests were used for comparative analyses. All results were analysed at the 95% significance level unless otherwise stated. In some cases, data were log transformed where knowledge of population distribution was not available or where relationships between variables had been previously demonstrated. When the effect of test agents on cell populations was undertaken, the relationship between control, unstimulated, cells and test, stimulated, cells was expressed as the ratio of stimulated to unstimulated cells. For assays that involved colourimetric measurements, the Relative Proliferation Index (RPI) or Stimulation Index (SI) was calculated by dividing the Optical Density (OD) of treated cells by the OD obtained from control, untreated cells. For radiometric assays, raw data is presented as mean counts per minute (cpm) ± one standard deviation (SD) and SIs were calculated as the raw cpm obtained after stimulation divided by the raw cpm of control cells cultured in media only. For assays where blank samples were included in the experimental design, results for blanks were subtracted from both control and test samples before statistical analyses were performed. Stimulation indices, relative proliferation values or percent of control values are used throughout this study to express the degree of proliferation. These methods were chosen to facilitate comparative assessments with previous studies. However, where control values are known to be unusually high, raw data is also presented to prevent misinterpretation of results.
2.3.3 Routine Identification of Leukocytes

2.3.3.1 Blood and Cell Isolates

Blood and cell smears were routinely prepared to assess purity of cell preparations isolated during this study. Blood films were prepared by placing a drop of cell preparation onto the bottom third area of a microscope slide and ‘pushing’ to create a blood smear that thinned to single cell level at the periphery of the blood film (Haen, 1995). Differential counts were undertaken in this area of the slide to ensure that cell morphology was as true as possible. Cell measurements were taken using slides prepared for differential counts and diameters were recorded using an eyepiece micrometer.

For immunohistochemical analysis and for the assessment of phagocytic capacity, two different techniques were used to prepare leukocyte slides. Leukocyte films were prepared as for blood films or when cell numbers permitted, discrete ‘drops’ of two microlitres of PBMC preparations (1×10⁶ cells/mL) were applied to microscope slides. After sample application, slides were air dried and fixed in 100% methanol for 3-10mins. Blood films or cell smears were stained with Diff Quik differential stain (Laboratory Aids Pty Ltd, NSW) according to manufacturer’s instructions. Alternatively, where greater staining contrast was desired, slides were stained with Giemsa Stain (Merck & Co. Inc.; NJ, USA) for 3mins followed by a rinse in tap water. Slides were preserved by mounting with Entellan mountant (Merck & Co. Inc.; NJ, USA) after a single dip in xylene. Where cells were isolated for specific purposes such as immunocytochemistry and oxidative burst analysis, cell smears were stained with different dyes that accentuated the reaction in question. These procedures are outlined in the detailed protocol of the individual assays.

2.3.3.2 Tissues

Paraffin-embedded tissues were sectioned at 4 or 6μm and deparaffinised through xylene and a graded alcohol series (Celis, 1994). Consecutive sections were subsequently stained with haematoxylin and eosin (H and E) (Bancroft and Stevens, 1990) and surveyed for staining characteristics, tissue architecture, cell distribution and signs of pathology.
2.3.3.3 Total Cell Counts and Viability

2.3.3.3.1 Total Leukocyte Counts

Total leukocyte counts were performed on anticoagulant-treated whole blood samples diluted 1 in 10 with Turk’s Solution (0.15% toluidine blue, 1% glacial acetic acid, 0.08% saponin). This solution lysed red blood cells and stained leukocytes to assist counting. Cell counts were determined microscopically using an improved Neubauer chamber haemocytometer under 400x magnification and expressed as total cells/mL of blood.

2.3.3.3.2 Cell Viability and Enumeration

The assessment of the viability of isolated cells was undertaken using trypan blue stain. Cell preparations were diluted 1:1 with trypan blue solution (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic) and inspected microscopically using an improved Neubauer chamber haemocytometer under 400x magnification. Trypan blue will only enter across the membranes of degenerated or dead cells (Doyle and Griffiths, 1998; Brousseau et al., 1999) and thus total cell numbers as well as the proportion of non-viable cells was determined simultaneously.

2.3.3.4 Differential Cell Counts

Differential counts were performed on at least 100 cells in an area of the blood smear where red blood cells were not overlapping. Cells were inspected for general morphology, nuclear structure, staining properties and characteristic attributes such as nuclear to cytoplasm ratio and the presence or absence of granules. Inspection of viable cells was also undertaken using Phase contrast microscopy and Differential Interference Contrast (DIC) microscopy to improve the resolution of membrane and cytoplasm morphology.

2.3.3.5 Electron microscopy

Transmission electron microscopy was used to identify the ultrastructural properties of blood leukocytes. Cells were inspected for indicators of synthetic activity such as rough endoplasmic reticulum (RER), prominent nucleoli, relative proportions of heterochromatin and euchromatin and the presence of Golgi bodies and mitochondria (Zucker-Franklin et al., 1981). Morphological characteristics such as cytoplasmic
margins, the numbers and types of granules and the presence of vacuoles and membrane inclusions were also recorded.

Isolated cells (Section 2.3.4) were rinsed twice in HBSS- and pelleted by centrifugation at 200g for 5mins. After washing, cells were suspended in Karnovsky’s fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde, 0.1M cacodylate, (pH 7.4)) for 1.5 hours, washed twice and stored in 0.1M MOPS buffer (3-(N-Morpholino) propanesulphonic acid) until further processed. The cells were BSA encapsulated and fixed in Karnovsky’s fixative for 12hours at 4°C after which time the cell pellet was removed and washed with 0.1M MOPS buffer. The buffer was decanted and the cell sample was treated with osmium tetroxide (OsO4) for two hours followed by treatment with 2% buffered uranyl acetate for one hour. The sample was dehydrated in an ethanol series to acetone and transferred to an acetone/resin mixture for 1 hour. After three 10min incubations in resin at 70°C, the samples were embedded and polymerised at 70°C for 18 hours.

Post-staining of 0.5μm thin sections was carried out with methylene blue. After identification of areas optimal for sectioning, ultra-thin sections were cut and mounted on copper grids and processed with lead citrate followed by uranyl acetate to accentuate ultrastructural elements using transmission electron microscopy.

2.3.3.6 Microscopy and Photography
A number of different microscopes were used in this study to record observations and to obtain photomicrographs. These included a CX40 compound light microscope (Olympus) fitted with an SC35 camera and an Axiophot 20 microscope (Zeiss) fitted with an MC80 camera. Inverted phase microscopy was performed on an Olympus CK2 microscope also fitted with an SC 35, Type 12 Camera. Transmission electron micrographs were obtained using a CM120 BioTWIN (Philips) transmission electron microscope (TEM) at 100kV or a CM10 TEM (Philips) at 80kV.

2.3.4 Isolation of Cells from Blood, Spleen and Lymph Nodes
A number of different protocols were employed to isolate and purify the various leukocyte populations studied in blood and lymphoid organs. All cell preparations
were subjected to treatments to remove red blood cells except where otherwise indicated. Since this was a common procedure employed for all cell populations, it is presented at the beginning of this section to avoid repetition.

2.3.4.1 Removal of Red Blood Cells (RBC)

In protocols that required purification of white blood cells, contaminating RBCs were removed using hypotonic lysis or treatment with a tris-buffered ammonium chloride solution (0.16M ammonium chloride, 0.01M tris(hydroxymethyl)aminomethane (Tris), pH to 7.3; RBC lysis solution). For experiments using hypotonic lysis, the buffy coat was washed once with appropriate media before the cell pellet was subjected to a 20s treatment with cold (on ice) sterile milliQ water. To restore tonicity, an equal volume of 2x PBS was added and the sample was immediately diluted 10 fold in appropriate media.

RBC Lysis Solution was also used to reduce RBC contamination and to improve the culture viability of cells. The buffy coat cell pellet was resuspended to 3mL with warmed (37°C) RBC lysis solution and incubated at 37°C in humidified air containing 5% carbon dioxide (CO₂) for 15mins. After this time, cell suspensions were diluted to 10mL with appropriate washing solution and centrifuged at 350g for 10mins at 18°C to recover cell pellets devoid of RBC. Cells were further washed in the appropriate media for individual experiments and suspended to the desired volume for analysis.

2.3.4.2 Cell Isolation from Blood

2.3.4.2.1 Peripheral Blood Mononuclear Cells (PBMC)

During this study, a number of cell isolation techniques were used in order to optimise recovery of cells from blood and tissues. Results presented in this work comprise those obtained from cells harvested by these various methods, so they are presented here in list form to allow easy identification throughout the text. Method a), density gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech; San Francisco) was the method of choice and was used in all cell isolation procedures unless otherwise stated. All blood or buffy coat samples utilised for density gradient centrifugation were diluted 1:1, 1:2 or 1:3 in HBSS, PBS or media as required before use.
a. Mononuclear Cells were isolated by density gradient centrifugation over endotoxin-free Ficoll-Hypaque or Histopaque 1.077 or 1.083 according to the method of Boyum (1964). Briefly, 4mL of blood preparation was layered over 2mL of density gradient medium in a 10mL sterile, polypropylene centrifuge tube and centrifuged at 18°C at 400g for 25mins. When large volumes of blood were available, 20 to 30mL of blood preparation was layered over 10mL of density gradient medium in 50mL sterile polypropylene centrifuge tubes. In all cases, a buffy coat formed at the interface of plasma and density gradient media and was removed using a sterile polypropylene transfer pipette into a new sterile tube.

b. Percoll (Amersham Pharmacia Biotech; San Francisco) solutions of densities between 1.04 and 1.10 were prepared by diluting stock Percoll with 1.5M sodium chloride (10x physiological strength) and sterile milliQ water according to manufacturer’s instructions. 2mL of each of the prepared solutions of known density were layered sequentially into a 10mL sterile polypropylene tube. Whole blood or buffy coats isolated from method a) or c) were diluted to 2mL in appropriate medium and layered over the top of the prepared discontinuous gradient. Tubes were centrifuged at 390g for 25mins at 18°C. Cells localised at gradient interfaces were collected with a sterile polypropylene transfer pipette.

c. Whole blood was centrifuged at 500g for 20mins at 18°C or 4°C if serum was to be recovered. A buffy coat of leukocytes formed at the serum/RBC interface and was removed using a sterile polypropylene transfer pipette.

For comparative species studies, human PBMC were isolated using ficoll-paque and sheep PBMC were harvested using the whole blood leukocyte buffy coat method. This method was selected as sheep RBCs do not easily form rouleaux (Banks, 1981) and thus the separation of leukocytes from RBCs when performed directly on whole blood was unsuccessful. Sheep PBMC were therefore isolated using a 2-step process that involved harvest of the buffy coat from whole blood followed immediately by density gradient centrifugation of the whole blood leukocyte preparation.
2.3.4.2.2 Monocytes and Monocyte-Derived Adherent Cells
Adherent cell populations were isolated by positive selection from PBMCs, which relied on the adherence of monocyte and monocyte-derived cells to the culture vessel. Mononuclear cell preparations were plated into tissue culture flasks and incubated with RPMI-1640 supplemented with 10% FBS or serum-free QBSF®-51 medium for between 24 hours and five days. After 24 hours, suspension cells were removed by aspiration and media was replaced. In some assays, lymphocyte conditioned serum-free media (LCSFM) was added in place of fresh media. For culture periods longer than three days, 50% of the culture media was removed and replaced with fresh media. LCSFM was prepared by recovering the media from 24 hour PBMC cultures by centrifugation of suspension cell cultures at 300g for 10 mins.

The viability and numbers of adherent cells were originally assessed by trypan blue staining which required removal of the adherent cells from the culture dishes. Due to significant losses and low cell yields during these processes, the viability of adherent cultures in later cultures were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining, since live cells produced a dark blue MTT formazan precipitate which was easily identified.

Purity of the macrophage suspensions was assessed by microscopic examination of Diff Quik (Lab. Aids, Australia) stained smears when cells were successfully recovered from culture dishes. Staining of adherent cells within the culture dish was performed with Giemsa stain to improve staining contrast.

2.3.4.2.3 Suspension Cells – Purification of Lymphocytes
The non-adherent fraction of PBMC preparations, otherwise known as suspension cells in this study, were recovered by aspiration of the media from tissue culture flasks incubated with PBMCs, followed by centrifugation at 200g for 5mins. Cells were subsequently washed twice with HBSS-.

Fractionation of lymphocytes using nylon wool columns
To further separate lymphocyte populations into T and B cells, a technique involving the filtration of lymphocytes through a nylon wool column was adopted that relied on the relative adherent properties of these two populations (Oppenheim et al., 1968;
Trizio and Cudkowicz, 1974). In eutherian systems, B cells with their surface immunoglobulin molecules are ‘sticky’ compared with T cells and will preferentially adhere to a substrate more strongly than T cells. This difference in physical properties can be used to remove B cells from a lymphocyte suspension and allows collection of the non-adherent T cell population as the ‘flow through’ fraction after application to a nylon wool column. Nylon wool lymphocyte separation columns were constructed from sterile polypropylene syringes (barrels removed) fitted with 3mm sterile tubing and clamp to control flow rate. 3mL (for less than 5x10^5 cells/mL) or 10mL (for > 5 x 10^5 cells/mL) syringe barrels were loosely packed with sterile nylon wool (Polysciences, Inc.; Warrington, PA) and washed thoroughly with prewarmed (37°C) media. Columns were filled with medium compatible with the assay to be performed, sealed with Parafilm and pre-incubated at 37°C in humidified air supplemented with 5% CO₂ for 30mins. After removal of medium, cells were added slowly to the top of the nylon wool column and allowed to run into the barrel. Media was added to the top of the column before the column was sealed with parafilm and incubated for a further 1.5-2 hours. After this time, non-adherent (T) cells were collected by passing 2 volumes of media slowly through the column and adherent (B) cells were recovered by mechanical agitation of the nylon wool residue. Wash media was removed by centrifugation at 350g for 7mins and cell pellets were resuspended in 500µL of medium. Cell counts and viability were assessed using trypan blue exclusion.

2.3.4.2.4 Polymorphonuclear Cell Isolation: Neutrophils, Eosinophils and Basophils

Wherever possible, whole blood collected for granulocyte isolation was anticoagulated using EDTA rather than heparin, since heparin is known to contribute to false positive results (Rose et al., 1992) in the Nitroblue Tetrazolium (NBT) Assay described in Method 2.3.7.4.4 of this study. The different techniques for the isolation of granulocytes all relied on the increased density of these cells compared with PBMC and are described below.

a) Granulocytes were recovered from the ficoll-RBC or histopaque-RBC interface layer after density gradient separation of whole blood. To remove residual RBCs, cells were subjected to hypotonic lysis as previously described (2.3.4.1).
b) Granulocytes were recovered using the dextran sedimentation technique that relies on the differential gravity sedimentation properties of leukocytes and RBC when mixed with dextran (Wright, 1988). After removal of the PBMC layer using ficoll-paque centrifugation in a 50mL centrifuge tube, residual ficoll was aspirated to the granulocyte/RBC interface. 10mL of plasma was added back to this residual layer and this suspension was gently mixed with sufficient 3% dextran T500 (MW 500 00; Pharmacia; Uppsala, Sweden) in sterile 0.85% sodium chloride solution to fill the tube to the 50mL mark. The centrifuge tube was gently inverted five times and then allowed to stand at RT for 20mins. During this time, RBCs sedimented to the bottom of the tube. The supernatant, containing granulocytes, was aspirated and centrifuged at 250g for 10mins at 4°C. The supernatant was discarded and the granulocyte cell pellet was subjected to hypotonic lysis to remove residual RBCs as as previously described (2.3.4.1).

c) A double layer density gradient technique using Histopaque 1.077 and Histopaque 1.119 (both obtained from Sigma; Missouri, USA) was used in an effort to isolate granulocytes in a single-step process and to avoid the necessity for RBC lysis. In this procedure, Histopaque 1.077 was layered over Histopaque 1.199 according to manufacturer’s instructions. Whole blood was layered above the density gradient media and the samples were centrifuged at 700g for 30mins at RT. Two distinct cell layers were formed in the tube after this process. Granulocytes were recovered from the layer nearest the bottom of the tube.

d) Percoll isolation using discontinuous density gradient centrifugation was performed as outlined in section 2.3.4.2.1b for the isolation of PBMC. The most dense layer (that nearest the base of the tube) was harvested for granulocyte collection.

With the exception of protocol (c), hypotonic lysis for the removal of residual RBCs was carried out on all harvested granulocyte preparations. Cell preparations were then washed twice with HBSS- to remove residual haemoglobin, RBC ghosts and remaining traces of density gradient media. Viability and cell numbers were determined using trypan blue exclusion as previously described. Granulocyte
preparations were routinely assessed for purity using the Diff-Quik stain as outlined in Section 2.3.3.1.

2.3.4.3 Cell Isolation from Spleen and Lymph Nodes
Immediately after euthanasia, organs were removed using sterilised instruments, sectioned into pieces no greater than 2cm, and placed directly into transport media consisting of HBSS containing double strength antibiotics. Samples were carried to the laboratory in an insulated cooler and were processed within two hours of collection. Tissues were placed onto a sterile petri dish and gently minced by teasing with forceps in the presence of PBS to release lymphocytes (Klaus, 1987). Cell suspensions were then further dissociated by pushing through a 60-mesh stainless steel sieve. Cells were transferred to a centrifuge tube and allowed to settle for 2mins in order to sediment large pieces of unwanted tissue and large cell clumps. Supernatants containing free cells were centrifuged for 10mins at 400g at 18°C and resuspended in washing media. The viable free cells were then separated from the remaining debris and dead cells by density gradient centrifugation over ficoll-paque density gradient media (1.077) as described in section 2.3.4.2.1. Cells were washed in media containing antibiotics, resuspended in the desired volume and counted by haemocytometer using trypan blue exclusion.

2.3.5 Phenotyping of Lymphocytes – Immunohistochemistry
Species cross-reactive antibodies were used to identify T and B cells in both peripheral blood and tissue lymphocytes of marsupial cells investigated during this study.

2.3.5.1 Cells
Cell smears or two microlitre drops of PBMC preparations (1x10^6 cells/mL) were applied to cleaned untreated or poly-l-lysine coated microscope slides and air dried before fixation. A number of different fixative solutions and protocols were trialled in this study. Cells were fixed in a) 100% methanol for 1 minute at RT, b) 50% acetone/50% neutral buffered formalin for 15s, c) 15% acetone in methanol for 10mins at RT and d) 50% acetone/50% methanol (v/v) for 15mins at -20°C. With the exception of slides prepared using method a) which were held at RT, leukocyte slides
were wrapped in aluminium foil and stored at -20°C until tested. All samples were brought to RT immediately before analysis.

PBMC slides were rinsed (x3) in deionised water, followed by a permeabilisation step of 10mins in a solution of 0.25% triton X-100 plus 5% dimethylsulfoxide in physiologically buffered saline. Samples were rinsed three times in deionised water for 5mins per wash to remove detergents. Endogenous peroxidase was blocked, with constant stirring, by a 15, 30 or 45min incubation in a solution of 0.3, 0.9 or 1.5% hydrogen peroxide in methanol, PBS or water. Samples were rinsed three times in deionised water, dipped in PBS and processed for immunostaining using the Vectorstain Elite peroxidase kit (Vector Laboratories; California, USA). Cells were blocked with 1/50, 1/20, 1/10, 1/5 and whole horse serum for 30 or 60mins in a humid container. Excess serum was removed from slides followed by a 30 or 60min incubation with the primary antibody at dilutions of 1/10, 1/30, 1/50 and 1/100. Samples were washed in PBS for 5mins followed by a 15 or 30min incubation with the secondary antibody, a 5min wash in PBS and a further 30min incubation with the Avidin-biotin complex. The excess complex was removed by a 5min PBS wash and the sections were immediately covered with diaminobenzidine (DAB) in urea hydrogen peroxide (Sigma Fas™ Tablet Set; Sigma; Missouri, USA). The reaction was monitored microscopically for the appearance of a brown precipitate and was stopped with deionised water at 5, 8 or 10mins. Slides were then counterstained with haematoxylin and mounted using Entellen® mountant (Merck; Darmstadt, Germany).

2.3.5.2 Tissues

Tissue samples were collected in 10% neutral-buffered formalin or Bouin’s fixative, transferred to ethanol and embedded in paraffin and routinely processed (Celis, 1994). Paraffin-embedded slides were sectioned at either 4 or 6μm, deparaffinised through xylene and rehydrated through a graded alcohol series. Immunohistological experiments using the Vectastain Elite kit were undertaken on consecutive sections. To unmask cross-linked antigens, tissue sections were treated with Target Antigen Retrieval Solution (Dako; California, USA) or Vector Antigen Unmasking Solution (Vector Laboratories; California, USA) using a microwave treatment at 1000W for 20mins. Samples were left to cool in antigen retrieval solution at RT for 20mins.
before sections were transferred to PBS for equilibration. Each step of the following protocol was preceded by a 5 min wash with PBS and was performed in a humidified container at RT.

Endogenous peroxidase in tissue sections was blocked with 0.3% hydrogen peroxide in methanol/PBS. Non-specific binding was minimised by a blocking step with 10% horse serum in PBS for 60 mins. Primary antibody in blocking serum was applied to sections at dilutions of 1/5, 1/10, 1/50 or 1/100 for 30 or 60 min. A universal biotinylated secondary antibody was applied to each section for 30 min followed by 30 min incubation with complex. DAB in urea-hydrogen peroxide was added to each section and incubated for 5-8 mins to develop the brown precipitate indicative of positive antigen reactivity. Sections were counterstained with haematoxylin, dehydrated through an alcohol series and xylene and mounted with Entellen mountant. Positive antibody localisation was indicated microscopically by a brown stain in the membrane or cytoplasm of cells. Negatively stained sections stained blue.

2.3.5.3 Antibodies
Antibodies to the T lymphocyte surface antigens, CD3 and CD5 were used to investigate the identity of lymphocyte subsets in peripheral blood cells of the tammar wallaby, Mala and potoroo species. Anti-CD5 antibody and a B cell antibody, anti-CD79b, were used to investigate the tissues of the Mala, Long-footed potoroo and Long-nosed potoroo. Monoclonal antibodies to CD5 (clone CD5/54), CD3 (clone PC3/188) and CD79b (clone B29/123) were kindly supplied as tissue culture supernatants by Dr. Margaret Jones of the Leukaemia Research Fund Immunodiagnostics Unit (Oxford, United Kingdom). A commercially available polyclonal rabbit anti-human CD3 T cell antibody (A452, Dako; California, USA) that reacts with the intracytoplasmic portion of the CD3 epsilon chain, was also used in this study for the staining of tammar wallaby lymphocytes. Details of antibody dilutions used in the testing of cells and tissues are summarised in Table 2.5.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCD3*</td>
<td>1/50, 1/100, 1/1000*</td>
<td>Pan T cell marker</td>
<td>Dako (California, USA)</td>
</tr>
<tr>
<td>mCD3*</td>
<td>1/10, 1/50</td>
<td>Pan T cell marker</td>
<td>Margaret Jones (LRF)</td>
</tr>
<tr>
<td>mCD5</td>
<td>1/10, 1/30, 1/50, 1/100</td>
<td>Pan T cell marker &amp; some B cells in selected mammalian species</td>
<td>Margaret Jones (LRF)</td>
</tr>
<tr>
<td>CD79b**</td>
<td>1/10, 1/50</td>
<td>B cell marker. Expressed after pre-B cell stage, lost prior to plasma cell stage</td>
<td>Margaret Jones (LRF)</td>
</tr>
</tbody>
</table>

* Lymphocyte testing only, **Tissues only, LRF Leukaemia Research Foundation, Oxford (UK), m monoclonal, p polyclonal.

**Table 2.5: Antibody Specificity and Source**

Negative control samples were run in parallel with all test panels and included replacement of the primary antibody with PBS, mouse IgG1 isotype controls for T cell antibodies, mouse IgG2b isotype controls for the B cell antibody and PBS in place of the secondary antibody.

**2.3.6 Acid-Fast Staining**

The composition of the cell wall of Mycobacterial species allows their identification using the Ziehl-Neelsen differential stain (Prescott et al., 1993). This method exploits the high levels of mycolic acids and other lipids found in the cell walls of these bacteria such that basic fuschin dye is retained and not decolourised by treatment with an acid-alcohol wash. Using this technique, the mycobacteria retain a red taint and can be readily identified as ‘acid-fast’ using light microscopy.

Cell and tissue samples obtained from Mala and potoroo species suspected to be infected with mycobacterial species were investigated for the presence of acid-fast micro-organisms using the carbol-fuschin technique (Dart, 1996). Paraffin embedded tissue sections were cut to a thickness of 6μm, deparaffinised in xylene, rehydrated through a graded ethanol series and rinsed in water. Cells smears were fixed in methanol, air dried and also rinsed in water. Both cell and tissue preparations were stained with Accustain Acid Fast Staining kit (Sigma Diagnostics®; Missouri, USA)
according to manufacturer’s instructions. Samples were treated with carbol fuchsin for 4mins, washed with deionised water and differentiated and counterstained with malachite green solution for 2mins. After washing in tap water, blotting and mounting, the acid-fast bacteria stained pink against a green background. Commercially purchased tissue sections containing mycobacterium species (Sigma; Missouri, USA) were included in staining protocols as a positive control.

2.3.7 In Vitro Cellular Assays
2.3.7.1 Proliferation Assays
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay was the principal assay used to assess cell proliferation in this study. The Tritiated Thymidine ([3H]-Thymidine) Incorporation Assay was also carried out on a select number of samples to validate the MTT assay and to provide comparative responses using an assay method previously employed by other marsupial researchers (see 1.5.6.2.2). Where cell numbers permitted, duplicate culture plates were prepared, cultured under standard conditions and processed using both the MTT and tritiated thymidine incorporation assays.

2.3.7.1.1 MTT Assay
The MTT assay measures the activity of living cells by cleavage of the tetrazolium ring by mitochondrial dehydrogenases (Mosmann, 1983). This assay is routinely used in human cell culture systems to assess cell viability both in individual cells as a deposit of reduced formazan precipitate, or as a spectrophotometric measurement for assessment of larger cell numbers. This assay may be used for detection of individual cell viability, indirect determination of cell numbers and for proliferation studies (Doyle and Griffiths, 1998). In this study of marsupial cell function, the MTT assay was optimised for use in both isolated PBMC and whole blood cultures.

MTT Standard Curve
Quantitation of cell number was determined indirectly by construction of a graph of the relationship between Optical Density (OD) of reduced MTT against viable cell number as determined using a haemocytometer. Stock PBMC suspensions were diluted in warmed (37°C) media to cell numbers between 5.5 x 10^5 cells/mL and 2.0 x 10^7 cells/mL. 100µL of each cell dilution was added to triplicate wells. 100µL of
media was added to each well to obtain a culture volume of 200μL. 20μL of stock MTT was immediately added to each well and the plate was incubated at 37°C in humidified air containing 5% CO₂ for 4 hours. The amount of formazan deposited within each cell was determined spectrophotometrically as described below for the MTT Assay.

**PBMC Assay**

The MTT assay as originally described by Mosmann (1983) was applied to both serum-supplemented and serum-free culture systems used in this study. After plating of the desired cell number, mitogens and bacterial LPS were added to wells at the concentrations and for the incubation times specified in Section 2.3.7.2. In serum-free systems, this protocol was modified to replace the original extraction solvent with that recommended by Hansen et al. (1989). After the incubation of cells with MTT for 4 hours under standard conditions as described above, a blue precipitate was visible at the base of the culture vessel. This MTT formazan product, formed as a result of the reduction of MTT by mitochondrial enzymes, was solubilised with an extraction solvent after centrifugation of the microtitre plate at 250g for 5mins and aspiration of the culture media. 100μL of 20% Sodium Dodecyl Sulphate/N, N-dimethylformamide extraction solvent (pH 4.7) was added to each well and the blue precipitate was dissolved on mixing with a microplate shaker for 10mins at 200rpm. The OD of dissolved formazan was recorded at 550nm using a BioRad Model 550 Microplate Reader (BioRad; California, USA). To decrease interference from unreduced MTT, background OD readings were referenced to 655nm as recommended by Gieni et al. (1995).

**MTT Suspension Assay**

As originally recommended by Mosmann (1983), initial MTT experiments were undertaken in serum-supplemented media systems and were performed by direct addition of a hydrochloric acid solubilisation solution (0.1N hydrochloric acid in methanol) to the culture vessels, without removal of residual media. This protocol was later modified to remove 100μL of culture medium before addition of 100μL of extraction solvent. Since cells cultured in serum-supplemented systems were less adherent to the base of the culture vessel than those cultured in serum-free medium, a number of changes to the MTT suspension assay protocol were trialled. These trials
included changes in the quantity of extraction solvent added (50µL, 100µL, 120µL and 150 µL) coupled with the removal of different quantities of culture media (50µL, 100µL, 120µL, 140µL, 150µL and 160µL) and changes to mixing times (10mins, 20mins, 30mins, 60mins) and mixing temperatures (RT and 37°C). Where media was removed from suspension assay plates, an additional centrifugation step was included to the protocol to sediment the cells at the base of the culture vessel to avoid accidental removal of suspension cells when media was removed. In some cases where non-adherent colonies were formed, MTT assays were performed in 1.5mL centrifuge tubes to allow a further centrifugation step to sediment cells and to facilitate removal of MTT supernatant without disturbing the cell pellet.

**Whole Blood Assay**

Volumes of whole blood (100µL, 50µL, 25µL, 20µL, 15µL, 10µL, 5µL, 4µL and 2µL) were added to microtitre plates after dilution in HBSS-, RPMI 1640 media or QBSF®-51 media. Test agents and the appropriate media were added to a total volume of 200µL. Samples were incubated for 24, 48, 72 and 96 hours at 37°C, 5% CO₂ and 100% humidity. After incubation, samples were centrifuged at 400g for 7mins and supernatants were removed, leaving the RBC and leukocyte pellet undisturbed. RBCs were either lysed with cold milliQ water followed by addition of equal volumes of 2xPBS or with RBC lysis solution. After addition of 200µL of media, 1/10 volume of MTT was added to samples and these were incubated for a further 4 hours under standard conditions. In some assays, no RBC lysis step was undertaken and in others, RBC lysis was performed after the MTT incubation step. In all cases, the OD of samples was read at 550nm and referenced to 655nm. When larger volumes of blood were sampled and no RBC lysis step was performed, optical density values were greater than 3.0 and a further processing step was added to the protocol. After the MTT incubation step, MTT formazan was dissolved by direct addition of 50µL or 100µL of extraction solvent followed by mixing for 10mins on a microplate shaker. After dissolution of the formazan product, known volumes of sample were removed and either read directly or were further diluted with 100µL of extraction solvent prior to reading.
2.3.7.1.2 $[^3]H$-Thymidine Incorporation Assay

100µL of cell suspensions or whole blood samples were added to 96 well microtitre plates. Mitogens and media were added to a total volume of 200µL. Plates were incubated for 48hrs at 37°C in a 5% CO$_2$ humidified atmosphere. After this incubation time, 1.0µCi $^3$H-thymidine (25Ci/mmol; Amersham Pharmacia Biotech; Sydney, Australia) was added to each well and the plates were incubated for a further 4 hours under standard conditions. To reduce errors associated with RBC quenching in whole blood assays, RBCs were lysed with RBC lysis solution prior to addition of thymidine. RBCs were sedimented by centrifugation at 250g for 3mins, supernatants were removed and 250µL of RBC lysis solution was added to each well. After incubation under standard culture conditions for 30mins, the plates were gently shaken and centrifuged at 250g for 3mins to sediment the leukocytes. The haemoglobin-containing supernatants were removed and samples were washed with PBS to remove residual traces of lysis solution. Both PBMC and whole blood samples were then processed according to the method of Burke et al. (1997). After centrifugation of the microtitre plate at 250g for 3mins, unincorporated $^3$H-thymidine was removed from each well with two washes of PBS for PBMC preparations and one wash for whole blood assays. Acid-soluble material was dissolved in 200µL of 5% Trichloroacetic Acid (TCA) and then removed by aspiration. Acid-insoluble material remaining in the wells was washed twice with ethanol and solubilised by a 30min incubation in 200µL of basic extraction solution (0.1M sodium hydroxide containing 2% sodium carbonate with 1% sodium dodecyl sulfate). After incubation, the well contents were transferred to scintillation vials containing 4.5mL of Starcsint scintillation fluid (Packard Biosciences, The Netherlands). The amount of thymidine incorporation into newly synthesised DNA was counted using a Tri-Carb 2100 Packard Liquid Scintillation Analyser (Canberra, Australia). For this assay, test panels included media only, control cells with no mitogen treatment and cells treated with mitogen. Data generated from these experiments are presented as mean counts per minute (cpm) ± one standard deviation (SD) from triplicate samples or as Stimulation Indices (SI) where the raw cpm obtained after mitogen stimulation was divided by the raw cpm of control cells cultured in media only. Occasionally, mean stimulation indices are expressed as percentage of control cells to facilitate comparisons between samples.
2.3.7.2 Proliferation Experiments

Optimisation of a range of cell culture parameters was undertaken to ensure culture conditions were compatible with the MTT Assay to be used for measurement of proliferation in this study. These included the type and quantity of media, the optimal range of cell numbers, the optimal mitogen concentration and incubation time as well as modifications in MTT assay protocol such as removal of culture medium before measurement of OD and the type and quantity of MTT solubilisation buffer. The following is a list of cell culture conditions optimised during this study:

2.3.7.2.1 Cell Numbers

A range of cell numbers were trialled during this study. Between $8.0 \times 10^4$ and $1.8 \times 10^5$ PBMC/well were assessed for their ability to support mitogen-driven proliferation.

2.3.7.2.2 Mitogen Concentration

Due to sample volume limits imposed by animal ethics considerations in this study, mitogen-driven proliferation assays were undertaken as a collection of small, discrete studies rather than as more expansive experiments that were able to test a large number of variables concurrently. The following is a summary of the mitogen levels used for the different experiments undertaken in this study. More detailed information is presented in the results section for each mitogen tested.

For whole blood assays, samples were treated with PHA at levels of 50μg/mL, ConA at levels of 5, 10, 25 and 50μg/mL and PWM at 50 and 63.5μg/mL.

For PBMC and lymphocyte culture assays, cells were treated with:

i) PHA at levels of 2.5, 5.0, 10.0, 20.0, 25.0, 50.0 and 100.0μg/mL

ii) ConA at levels of 5.0, 10.0, 20.0, 25.0μg/mL

iii) PWM at levels of 1.0, 2.0, 2.5, 5.0, 10.0, 20.0, 25.0 and 33.0μg/mL

iv) PMA at levels of 10, 25, 50, 75, 100, 150 and 200ng/mL

v) Ionomycin at levels of 50ng/mL, 1 and 5μg/mL

vi) LPS at levels of 2.5, 5.0, 7.5, 10.0, 50.0, 100.0, 250.0 and 500μg/mL.
2.3.7.2.3 Culture Volume
All whole blood assays were undertaken with total culture volumes of 200μL. PBMCs were cultured in 96-well microtitre plates or 1.5mL centrifuge tubes in volumes of 150μL, 200μL and 250μL.

2.3.7.2.4 Incubation Time
Whole blood, PBMC and purified lymphocyte cultures were incubated for 24, 48, 72, 96 and/or 120 hours to determine the optimal culture duration. Time course experiments were undertaken when sufficient cell numbers were available. All samples were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

2.3.7.3 Cell Culture Supernatants
2.3.7.3.1 Preparation
Lymphocytes were isolated from peripheral blood, spleen and lymph nodes as described in Section 2.3.4.2.1a and dispensed into 24 or 12 well tissue culture plates. Cells were stimulated with optimal doses of mitogens (25μg/mL PHA, 5μg/mL PWM in serum-free cultures and 10μg/mL in RPMI + 10% FBS) for 6-24 hours. Microtitre plates were spun at 300g for 10min to sediment cells and supernatants were removed. Cells were washed three times with HBSS or serum-free media to remove residual mitogen and fresh serum-free media containing antibiotics was added back to the wells. Microtitre plates were incubated for a further 24-48 hours before collection of the cell culture supernatants by centrifugation at 500g for 10mins.

2.3.7.3.2 Processing of Supernatants
Supernatants harvested from mitogen-stimulated lymphocytes and control cells were filter sterilised through a 4mm Millex®-GV Syringe driven Filter Unit fitted with a 0.22μm Durapore low-protein binding polyvinylidene fluoride membrane (Millipore; MA, USA).

Various dilutions (1/5, 1/10, 1/20 and 1/50) of whole supernatants were tested for their proliferation properties against freshly isolated PBMC. Fractionation of supernatants from mitogen treated and control wells was performed on those supernatants where bioactivity was identified using the MTT proliferation assay. Two
crude fractions were obtained by the centrifugation of whole supernatants through Nanosep spin columns (Pall Gelman Laboratory; MI, USA) fitted with a membrane to retain molecules with a molecular weight of greater than 50 000Da. Product retained on the filter membrane, the retentate, was diluted with 125 μL of filter-sterilised serum-free media and further sterilised before storage at -20°C. The filtrate was recovered in its original volume and spun over a filter membrane to exclude molecules greater than 30 000Da or 50 000Da, depending on the availability of membranes. The retentate was resuspended in 125μL of serum-free (SF) medium. Both the retentate (>10kDa<30kDa or 50kDa) and the filtrate (<10kDa) were collected, filter sterilised and frozen at -20°C until required. All fractionations were carried out on control samples treated in the same manner. Both fractions were assayed for bioactivity on PBMC using the MTT assay (2.3.8.1.1) in order to localise proliferative agents identified in preliminary screenings using unfractionated supernatants.

2.3.7.4 Antimicrobial Responses
Marsupial phagocytic cells were assessed for their ability to produce antimicrobial responses in vitro. This study investigated the capacity of both mononuclear and polymorphonuclear cells to respond to phagocytic stimuli and to produce both oxidative and non-oxidative cellular responses.

2.3.7.4.1 Polarisation Assays
Changes in the morphology of phagocytic cells, particularly the granulocytes, can be used to determine the ability of cells to detect signals in their environment (Wilkinson and Haston, 1988). These changes, collectively termed polarisation responses, were used to assess the effects of various soluble and particulate treatment agents on the tammar wallaby granulocytes. Granulocytes were isolated (2.3.4.2.4) and kept at RT or stored on ice (as indicated) until further use. Polarisation responses were assessed according to the method of Haston and Shields (1985). Stock cells were diluted to 2x10^6 cells/mL in 10mM 3-(N-Morpholino) propanesulphonic acid (MOPS) in HBSS. 1mL of this cell preparation was added to 1mL of 10mM MOPS containing the polarising test agent and this suspension was incubated in a 10mL polypropylene centrifuge tube at 37°C for 30mins. Equal volumes of 2.5% glutaraldehyde 10nM
MOPS were added to each cell preparation and incubated for a further 10mins. The fixed cells were washed in 10mM MOPS and resuspended in approximately 300µL of media for microscopic examination. 200 cells were examined by phase contrast microscopy and scored by morphology into polarised or unpolarised cells where a polarised cell was defined as any cell that did not have a spherical morphology. The degree of polarity was assessed by increase in cell length as determined by the largest diameter measured in micrometres (µm).

2.3.7.4.2 Chemotaxis Assays
Chemotaxis is the directional movement of cells under the influence of a chemical substance. The ability of cells to recognise and respond to substances released by cells or foreign invaders determines the ability of the effector cells to home to the infection site and deal with the immune assault (Wilkinson and Haston, 1988). In a modification of the Boyden chamber technique (Boyden, 1962), a 48 well tissue culture plate was used to capture migrating granulocytes through a 3µm disposable filter-insert placed above each well. Medium containing the test agent was placed in the bottom well. The filter was fitted directly over the base well to ensure complete wetting of membrane that created an effective seal for the duration of the assay. Granulocytes suspended in HBSS were placed in filter units at 5 x 10^5 cells/well and incubated for 1 hour, 2hours or 18hours with and without chemotactic test agents to allow complete migration through filters to the bottom of the wells. Controls to assess the effects of gravity and random cell migration were included in these assays, using HBSS as the control medium. Cells that successfully migrated completely through the filter and into the tissue culture well were quantified by direct counting using an inverted phase microscope or by indirect OD measurement by the MTT assay described in Section 2.3.7.4.2 (as for the PBMC assay).

2.3.7.4.3 Phagocytosis Assays
To assess the phagocytic capacity of marsupial leukocytes, inert latex particles of 1.1 and 3.0µm diameter, non-viable yeast (3-5µm; zymason) and viable gram positive (Staphylococcus aureus and S. xylosus) and gram negative bacteria (Escherichia coli) were all used in phagocytic assays undertaken in this study. Bacterial suspensions were prepared for use at 10^7 cells/mL.
Phagocytic assays were performed by one of the three following methods:

1. Whole blood samples and isolated cells were separately incubated with phagocytic test particles in sterile 1.5mL centrifuge tubes or 3mL polypropylene tissue culture tubes. 500µL of whole blood samples were diluted 1:1 with media and incubated with 20µL of zymosan, latex bead or bacterial suspensions. Isolated cells were resuspended at 10^6 cells/mL in media and 1mL was incubated with 10, 20 or 50µL volumes of non-opsonised or serum-opsonised zymosan, latex bead or bacterial suspensions. These preparations were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for one, two, three, four or 24 hours. After incubation, cell suspensions were gently centrifuged at 150g for 5mins to sediment the cells and 800µL of the suspension media (containing many of the uningested particles) was removed. For whole blood samples, RBCs were hypotonically lysed according to the method outlined in Section 2.3.4.1. and resuspended in 200µL of HBSS-. After gentle resuspension of cells, slides were prepared to allow inspection of the ingestion of particulate material. 2 - 5µL aliquots of the remaining cell suspension was either smeared or dropped onto an ethanol cleaned microscope slide. Slides were air-dried, fixed in methanol for 3mins and stained with modified Harris Hematoxylin Solution and counterstained with Accustain™ modified Eosin Y Solution (Sigma; Missouri, USA) or stained with crystal violet to allow more contrast between the zymosan particles and the cell cytoplasm. Giemsa stain was used on whole blood preparations to facilitate cell differentiation. The number of ingested particles per cell was counted using light microscopy. Phagocytosis was scored based on the number of cells that had ingested particles as well as the number of particles ingested per cell.

2. Cells were applied to pre-washed cover slips in 35mm culture dishes and allowed to adhere for 30mins. Suspension cells were rinsed clear of the culture vessel by two washes with HBSS-. Latex beads were added to the wells together with 1mL of HBSS- and incubated for a further 3 hours. Excess beads were washed from the cover slip surface by repeated rinsing with PBS until microscopic inspection revealed no free beads were present in suspension. Cover slips were air dried and routinely stained as for method (1) above.
3. Cells were isolated by adherence to the base of the tissue culture vessel and rinsed free of suspension cells by two washes with HBSS-. Latex and zymosan particles were added according to method (2) above. Samples were inspected during and after particle ingestion by inverted phase microscopy and upon completion of phagocytic assay, were air dried and stained in situ with Giemsa stain for 3mins. The stain was removed by two washes with water and the sample air dried before cell ingestion was quantified as outlined in method (1) above.

Cells were scored as positive (+) or negative (−) for ingestion and the numbers of ingested particles were counted using light microscopy at 1000x magnification.

2.3.7.4.4 Nitroblue Tetrazolium (NBT) Assay
Assessment of oxidative responses was undertaken using a number of different protocols. The NBT assay detects the presence of reactive oxygen intermediates (ROI) by formation of a blue precipitate within test cells formed by the reduction of Nitroblue tetrazolium (NBT) to formazan by molecules such as the superoxide anion and hydrogen peroxide.

Blood collected for the NBT slide assay was collected in EDTA-coated blood collection tubes as heparin has been implicated in false positive results in this assay (Rose et al., 1992). Stock solutions of NBT were prepared by dissolving nitroblue tetrazolium chloride in media or balanced salt solution to a final concentration of 5mg/mL. This solution was filter sterilised through a 0.22μm Nalgene filter to remove insoluble particulates.

NBT Slide Test
Granulocytes were isolated, washed with buffered salt solution and applied (~200μL drops) to poly-l-lysine coated slides to facilitate cell adherence. Slides were incubated in a humidified container at 37°C for 30-60mins. Samples were carefully washed with PBS and while still moist, treated with 200μL NBT +/- stimulating agent and returned to the humidified container for 30, 45 or 60mins at 37°C unless otherwise stated. Stimulating agents used in this study included PMA at 10μg/mL.
and LPS at 10μg/mL and 100μg/mL. NBT solutions were removed from slides by washing with PBS. Slides were fixed with methanol for 30s and counterstained with 0.5% saffranin for a further 30s to assist with visualisation of cell morphology. Samples were air-dried and inspected by light microscopy for evidence of blue precipitate within the cells.

**NBT Colourimetric Assay**

Cells suspended in balanced salt solution were plated at between $1 \times 10^5$ and $5 \times 10^7$ cells/well into 96 or 48 well microtitre plates. Treatment agents, NBT to a total concentration of 1 or 2μg/mL and media to 200, 250 or 300μL total volumes were added to the wells depending on the conditions of the specific assay. NBT formazan precipitate, produced by the reduction of NBT by reactive oxygen species, was solubilised according to the method of Rook et al. (1985). After incubation of cells with NBT, microtitre plates were centrifuged at 350g for 5mins and the supernatant removed. The culture plates were rinsed three times in methanol to remove unreduced NBT and allowed to air dry at RT. NBT formazan was then solubilised by addition of 120μL/well of 2M potassium hydroxide followed by 140μL/well of dimethylsulfoxide. The contents of each well were mixed by gentle pipetting and subsequently read on an enzyme-linked immunosorbent assay (ELISA) platereader at 655nm against control cells and a blank containing NBT and no cells. Treatment agents used to trigger the respiratory burst included LPS at 10, 50 and 100μg/mL, PMA at 1μg/mL and fMLP at $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$M.

### 2.3.7.4.5 Superoxide Anion Assay

To investigate the specificity of the granulocyte response for the production of superoxide anions, cells were treated with superoxide dismutase (SOD) after the method of Wolach et al. (1998). This enzyme acts to prevent the production of the superoxide anion, so that identification of the reactive oxygen species may be confirmed. Detection of the effectiveness of the suppression of this anion was undertaken in conjunction with the Ferricytochrome C assay, an assay that also detects the presence of oxidative burst products. 100μL of granulocyte preparations at a density of $5 \times 10^6$ cells/mL in HBSS were placed in triplicate into wells of a 96-well microtitre plate and pre-incubated with or without 170U/mL superoxide dismutase.
(stock at 1000U/mL; 3350 units/mg solid) for 5 min at 37°C. 60μM of ferricytochrome-C (stock at 600μM in HBSS-) was added and the oxidative response was initiated by the prompt addition of 100μg/mL LPS or 1μg/mL PMA. Reaction mixtures were incubated for 10 mins at 37°C and stopped by placing the trays in melting ice. Microtitre plates were centrifuged at 300g for 10 mins at 4°C and 100μL of the supernatant was removed to a new tray. Production of respiratory burst products in the test wells was assessed by measurement of the OD of triplicate samples measured at 550nm and referenced to 655nm. In wells that contained superoxide dismutase, this response was suppressed when the superoxide anion was responsible for the respiratory burst response. The production of the superoxide anion was also assayed in monocyte-derived adherent cells using this same protocol.

2.3.7.4.6 Peroxidase Activity
The ability to produce hydrogen peroxide may be indirectly assessed by testing for the peroxidase enzyme. The presence of peroxidase enzymes can be detected by the formation of a light brown pigment that develops when 3,3′-diaminobenzidine tetrachloride (DAB) is oxidised in the presence of hydrogen peroxide (H₂O₂) (Edwards, 1994) according to the following reaction:

\[
\text{DAB} + \text{H}_2\text{O}_2 \xrightarrow{\text{peroxidase}} \text{Oxidised DAB (brown pigment)}
\]

Methanol fixed cell smears were treated with the Sigma Fast™ DAB tablet set according to the manufacturer’s directions and were counterstained with Accustain™ modified Harris Hematoxylin Solution (Sigma; Missouri, USA), dipped once in xylene and mounted with Entellen mounting medium. The presence of the peroxidase enzyme was indicated by a brown stained region against a blue background.

2.3.7.4.7 Nitric Oxide Assay
The production of NO was measured indirectly by assaying for the presence of nitrite (NO₂⁻) using the Griess reagent (Green et al., 1982). Units of NO₂⁻ were estimated from a standard curve prepared from dilutions of 100μM sodium nitrite from between 0 and 100μM in increments of 10μM. Unless otherwise specified, nitrite accumulation was measured in 24 hour supernatants of cell cultures with and without
the treatment agents LPS at 10 and 100μg/mL and PMA at 1μg/mL. After
granulocytes were suspended in HBSS-, 5 x 10⁶ granulocytes/well were placed into 96
well microtitre plates and stimulated with PMA and LPS for 30mins, 60mins or 24
hours. At the completion of the desired incubation time, cell culture supernatants
were harvested and clarified by centrifugation at 14000rpm for 2mins. One hundred
microlitres of supernatants were mixed with an equal volume of modified Griess
reagent. The mixture was incubated at RT for 10mins after which the absorbance of
each well was measured at 540nm. The production of NO was also assayed in
monocyte-derived adherent cells using this same protocol.

2.3.7.4.8 Antimicrobial Protein Assays
i) Granule isolation and preparation of granule extract
Granules were isolated from neutrophils and eosinophils according to the method of
Gennaro et al. (1983). Granulocytes were suspended in 0.34M sucrose solution and
disrupted in a homogeniser with pestle by 50-100 strokes to ensure cell disruption.
Unbroken cells and nuclei were sedimented by centrifugation at 250g for 10mins.
The granule population was collected in the resulting supernatant by centrifugation
for 10mins at 2°C and 10 000g. Granule preparations were stored at -80°C until acid
extraction was performed on pooled samples.

ii) Acid extraction, concentration and dialysis of granule proteins
Granule pellets were extracted at 2-4°C with 10 volumes of 0.2M sodium acetate (pH
4.0) for 2.5hrs with continuous stirring. The insoluble material was sedimented at
20000g for 30mins and re-extracted with 5 volumes of 0.2M sodium acetate (pH 4.0).
The granule extracts were then subjected to centrifugal filtration through molecular
weight cut-off (MWCO) spin columns (Pall Gelman Laboratory; MI, USA) of 1000K,
100K, 50K and 3K. Both the filtrate and retentate from the 50K and 3K spin columns
were collected and dialysed for 24hours at 4°C against large volumes of 0.2M sodium
chloride in 10mM sodium phosphate (pH 7.0) in cellulose membrane tubing
(benzyolated cellulose tubing with nominal cutoff <1200; Sigma; Missouri, USA)
until the pH of the dialysed material was 7.0.
iii) Bactericidal Assays

Stock cultures of *E. coli* and *S. aureus* were grown in Luria Broth (LBroth) to an optical density of 0.6. Duplicate samples of 100μL of 1/10⁵ dilution of culture containing between 10³ and 10⁴ colony forming units were added to 1.5mL microcentrifuge tubes containing 0.9mM calcium chloride in PBS and 5μg of test fraction or dialysis solution in PBS as a control. Centrifuge tubes were incubated with shaking at 37°C and 150rpm for 40mins. 50μL quantities of each sample were plated in duplicate onto nutrient agar plates and the number of colony forming units were enumerated after incubation at 37°C for 18-24hrs.

2.3.7.5 Protein Detection
2.3.7.5.1 Protein Assay

The total protein content of each granulocyte fraction was determined colourimetrically using the Coomassie® Plus Protein Assay Reagent Kit (Pierce; Illinois, USA) following the Microplate Protocol according to manufacturer’s directions. Dilutions of granule fractions were prepared for analysis by adding 10μL of test sample to 140μL of HBSS-. 150μL of BSA standards containing 0, 1, 5, 10, 15, 20 and 25μg/mL were used to construct the standard protein curve. Two blank solutions were also included that contained HBSS- and the dialysis control solution. All samples were placed into a 96 well microtitre plate and 150μL of Coomassie Plus Reagent was added to each well. The microtitre plate was mixed for 30s and absorbance was measured at 595nm. A standard curve of absorbance versus protein concentration was constructed and used to estimate the total protein concentration of each of the granule fractions.

2.3.7.5.2 Polyacrylamide Gel Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) was used to confirm the presence of the desired molecular weight components present in samples after MWCO spin columns were used to fractionate both mitogen culture supernatant fractions and granule fractions obtained from the acid extraction of granulocytes. 10-20μL of each sample was added to an equal volume of denaturing loading dye (0.0625M Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol, 0.01%
bromophenol blue) and heated for 15mins at 55°C. Samples and a commercially purchased Kaleidoscope molecular weight marker (BioRad) were loaded into the wells of either gradient gels (4-20%) or 12% polyacrylamide separation gels and electrophoresed in running buffer (25mM Tris, 192mM glycine, 0.1% SDS) at 150-200V for 30-60mins. Gels were stained with coomassie protein stain and destained with 10% acetic acid. In order to detect low quantities of proteins present in some fractions, gels were also stained with Silver Stain Plus (Bio-Rad Laboratories; California) according to manufacturer’s instructions. Gels were inspected for the absence of bands ≥ 100kDa to ensure that spin columns had removed any excess mitogen present in PHA and Con A activated PBMC cultures and that the larger molecular weight components of granules had been removed.

2.3.8 Molecular Characterisation of Immunoregulatory Molecules
In this study, partial cDNA sequences were obtained from a number of marsupial cytokine genes by application of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) methodology. This technique was used to amplify target regions of complementary DNA from blood or tissue samples obtained from the tammar wallaby, the Mala and the Long-footed and Long-nosed potoroo species. For each cytokine, areas of amino acid consensus within the coding regions of a broad range of species were inspected for regions containing the least redundant amino acids. Oligonucleotide primers were then designed to these regions. Where ambiguity was present in the nucleotide consensus sequence, the nucleotide in question was substituted with that of the tammar wallaby or brushtail possum as available. The PCR fragments amplified using these primers were sequenced and compared with known cytokine genes in order to determine the level of similarity between products identified in this study and those previously identified in other species.

2.3.8.1 RNA isolation
Blood and tissue samples used as a source of ribonucleic acid (RNA) were collected as described (2.3.1.4) and stored frozen at -80°C until required. RNA was extracted from harvested blood cells and tissue samples using Tri-Reagent (Sigma, Missouri, USA) according to manufacturer’s instructions. This product is a monophase solution that contains a mixture of guanidine thiocyanate and phenol that allows the simultaneous separation of RNA and DNA. It was chosen since many of the samples
utilised in this study are rare and this reagent provided the opportunity to collect as much material as possible from a limited sample base. Briefly, samples were treated with Tri-Reagent to dissociate tissue and inactivate nucleases, extracted with chloroform and treated with isopropanol to precipitate the RNA. An extra isopropanol fractionation step was added to reduce the quantity of contaminating genomic DNA. Isolated RNA was washed twice in 70% ethanol and the resulting RNA pellet was air dried for 15mins. The isolated RNA was dissolved in nuclease-free water and stored at -80°C for later use.

2.3.8.2 cDNA synthesis

cDNA was prepared using a commercially available Reverse Transcription Kit (Reverse Transcription System, Promega; USA) according to the manufacturer's instructions. Briefly, RNA from a number of sources was incubated with Avian Myeloblastosis Virus reverse transcriptase enzyme (AMVRT), ribonuclease (RNase) inhibitor, deoxynucleotidetriphosphates (dNTPs), buffer, magnesium chloride (MgCl₂), oligo dT primers and nuclease-free water at 42°C for 60mins, followed by a 5min incubation at 99°C to inactivate the AMVRT enzyme. Prepared cDNA was stored at -20°C until further use.

2.3.8.3 Glucose-6-phosphate dehydrogenase (G6PD)

In order to validate the integrity of prepared cDNA, a housekeeping gene, Glucose-6-phosphate dehydrogenase, was amplified from the RNA derived cDNA. G6PD is an oxidoreductase enzyme that plays a role in the oxidation of glucose via the pentose phosphate pathway and has a high level of coding sequence similarity between eutherian and metatherian species (Loebel et al., 1995). Using G6PD primer sequences designed from the sequence of a metatherian mammal, the wallaroo (M. robustus), a cDNA product of 1131bp was amplified when cDNA was successfully prepared from RNA as outlined in section 2.3.8.2.

2.3.8.4 Primer Design

Amino acid sequences for IL-1β, IL-10 and TNF-α were aligned against the relevant cytokine sequences from a representative range of species and the regions of greatest amino acid similarity were identified using the Genetics Computer Group (GCG)
package (Wisconsin) accessed through the Australian National Genomic Information System (ANGIS) (Figures 2.1, 2.3, 2.5). Once identified, the nucleotide sequences within this region were also aligned to facilitate selection of the most conserved sequence for oligonucleotide primer synthesis (Figures 2.2, 2.4, 2.6). Where low similarity was evident, the nucleotides in question were substituted with those from the same region in tammar wallaby or possum genes. The GCG ‘Pileup’ and ‘Pretty’ programs were accessed through ANGIS and used to display text sequence alignments presented in this work.

TNF-α primer set one (TNFCPF and TNFCPR) was donated by Dr. G. A. Harrison. This primer set was previously used to successfully amplify TNF-α from a tammar wallaby mammary-associated lymph node Rapid Amplification of cDNA ends (RACE) library (Harrison et al., 1999). TNFCPF and TNFCPR were designed from an alignment of mainly eutherian consensus sequences with the inclusion of one marsupial sequence from the brushtail possum. This primer set proved unsuccessful in an early attempt to amplify TNF-α from both potoroo species and the Mala under the PCR conditions used. After inspection of a new alignment of TNF-α coding sequences that included the tammar wallaby sequence as well as the brushtail possum sequence (Figures 2.1, 2.2), the forward primer (TNFCPF) was retained and reverse primer was redesigned (TNFPR3) to include nucleotide sequence data common to both marsupial species.

Table 2.6 lists the identities, size and sequence for all primers used successfully in the polymerase chain reaction to amplify partial cDNA sequences in this study.
<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide (identity &amp; size)</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TNFCPF 25bp</td>
<td>TTGCTGGGGCCACCCTCCTCTTCTG</td>
</tr>
<tr>
<td></td>
<td>TNFCR3 35bp</td>
<td>TGGGAAGACACCACCTAGGTAGATTTGGCTCA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL1BF2 20bp</td>
<td>TCTTTCAAGAAGAACCATC</td>
</tr>
<tr>
<td></td>
<td>IL1BR 27bp</td>
<td>TTGGGAAGTGCTGATGTACCAGTTGGG</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL10F 29bp</td>
<td>ACCTGGGCTGCCAGGCTTGTCAGAGATG</td>
</tr>
<tr>
<td></td>
<td>IL10R 29bp</td>
<td>TCAAAATCCCCCATTGCTTTTGTAGACTCC</td>
</tr>
<tr>
<td>TYPE 1 IFNs*</td>
<td>IFNt1F1 30bp</td>
<td>TGTCCTCCAT/CGAGATGA/CTC/GCAGCAGAC/TCT</td>
</tr>
<tr>
<td></td>
<td>IFNt1R1 30bp</td>
<td>GATTTCT/CA/GCTCTGACAACC/TTCCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA/GCA</td>
</tr>
<tr>
<td>G6PD</td>
<td>G6PDEx5F 30bp</td>
<td>CAGGCCAACCGCCTTTCTACCTGGCCTTG</td>
</tr>
<tr>
<td></td>
<td>G6PDEx13R 27bp</td>
<td>CCACCTTGTAGGTGCCCTCATACTGGAA</td>
</tr>
</tbody>
</table>

*Primers designed and supplied by Dr. G. A. Harrison, UWS

Table 2.6: Oligonucleotide Primer Sequences for Cytokines and the Housekeeping Gene, G6PD.
2.3.8.5 Polymerase Chain Reaction (PCR)
cDNA from various sources was used as the DNA template for the polymerase chain reactions performed in this study. PCR reaction cycling conditions for all amplifications included 5μL cDNA, 10pmol primers, 200μM dNTPs, 25mM MgCl₂, 2.5U Taq polymerase and nuclease-free water to 50μL. Original PCR products were amplified at 45°C to facilitate screening. Once a product was obtained, the PCR was repeated on new cDNA at the higher temperature of 50°C to increase specificity. Table 2.7 lists the primer sets, reaction conditions and the expected product sizes for successful PCR reactions used in this study.

<table>
<thead>
<tr>
<th>Target Sequence</th>
<th>Primer Sets</th>
<th>Expected Product Size (base pairs)</th>
<th>PCR conditions denature/(anneal,denature/extend) /extend</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TNFCPF/TNFCR3</td>
<td>478</td>
<td>94°C for 1min / (94°C for 30s, 50°C for 50s, 72°C for 1min) x 35 / 72°C for 5mins</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL1BF2/IL1BR</td>
<td>464</td>
<td>94°C for 1min / (94°C for 30s, 45°C for 50s, 72°C for 1min) x 35 / 72°C for 5mins</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL10F/IL10R</td>
<td>255</td>
<td>94°C for 1min / (94°C for 30s, 45°C for 50s, 72°C for 1min) x 35 / 72°C for 5mins</td>
</tr>
<tr>
<td>Type I IFNs</td>
<td>IFNt1F1/IFNt1R1</td>
<td>265-280</td>
<td>94°C for 1min / (94°C for 30s, 45°C for 50s, 72°C for 1min) x 35 / 72°C for 5mins</td>
</tr>
<tr>
<td>G6PD</td>
<td>G6PDEx5F/G6PDEx13R</td>
<td>1131</td>
<td>94°C for 1min / (94°C for 30s, 50°C for 50s, 72°C for 1min) x 30 / 72°C for 5mins</td>
</tr>
</tbody>
</table>

Table 2.7: PCR Conditions for the Amplification of Partial cDNA sequences from Marsupial Tissues.
2.3.8.6 Gel Electrophoresis and PCR Product Purification

PCR products were electrophoresed through a 1.5, 2.0 or 2.5% agarose gel at 80V for 60mins using a Tris-borate EDTA (TBE) running buffer (89mM Tris, 89mM borate, 2mM EDTA; Smith, 1996). Agarose gels were stained in ethidium bromide solution (10μg/mL) for at least 30mins before visualisation using ultra violet light. In some experiments, ethidium bromide was added directly to the agarose gel.

PCR products of the required size were excised from agarose gels and purified using the Bresa-Clean DNA purification kit (Bresatec, Australia). This protocol relies on binding of target DNA to a silica matrix and subsequent recovery of purified DNA by elution with nuclease-free water. The Bresa-Clean kit was used according to manufacturer’s instructions with the following modifications: 1. A 10min ice-incubation step was added after addition of the bresa-bind silica matrix to facilitate DNA binding and 2. Extra washes of silica-bound DNA were included to ensure complete removal of contaminating buffer solutions (personal communication Dr. G. A. Harrison, UWS).

2.3.8.7 Cloning and Sequencing of PCR Products

DNA fragments of the desired size were cloned into the pGEM-T Easy Vector to facilitate the isolation of sufficient DNA for sequencing. PCR fragments were ligated into the pGem easy vector according to manufacturer’s instructions. JM109 competent cells were transformed and grown in the presence of LBroth for 1.5 hours before plating onto LB Agar containing ampicillin, Isopropyl-β-D-Thiogalactopyranoside (IPTG) and 5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside (X-Gal). After overnight incubation at 37°C, colonies were isolated based on blue/white screening (Sambrook et al., 1992). White colonies were inoculated into LBroth containing 100μg/mL ampicillin and grown for 16 hours in order to obtain sufficient material for future procedures. Mini-plasmid preparations were carried out using an alkaline lysis according to the method of Zhou et al. (1990).

Clones were screened for successful insertion of desired DNA fragments by performing an enzyme digest on the isolated plasmid DNA. The sequence for the
restriction enzyme, Eco R1, flanks the insert region of the p-GEM®-T vector. Treatment of the isolated plasmid DNA with this enzyme excises any DNA insert that was cloned into this vector. Hence, the size of the cloned product can be verified. Where verification of cloning success was required, plasmid DNA, Eco R1, Buffer H and nuclease-free water were incubated in a 1.5mL centrifuge tube for 2 hours at 37°C. Digestion products were visualised using ethidium bromide after products were electrophoresed on a 2.0% TBE agarose gel.

2.3.8.8 Sequence Analysis
Plasmid DNA was automatically sequenced by the Australian Genomic Research Facility (University of Queensland, Brisbane) using SP6 and T7 primers that flank the insert region of the p-Gem vector. Electrophoretograms resulting from this sequencing reaction were inspected for integrity, the sequences were trimmed of excess vector sequence and the forward and reverse sequence information was aligned to verify nucleotide data. The BLASTX program (Gish and States, 1993 and Altschul et al., 1990) accessed through ANGIS was used to determine the similarity of the returned sequence with those held in the Genbank database.
<table>
<thead>
<tr>
<th></th>
<th>cow</th>
<th>goat</th>
<th>human</th>
<th>cat</th>
<th>dog</th>
<th>horse</th>
<th>pig</th>
<th>mouse</th>
<th>woodchuck</th>
<th>rabbit</th>
<th>possum</th>
<th>tammar</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mstksmdrv elaeelvsek agggqgqssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>goat</td>
<td></td>
<td>...........</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>human</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cat</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dog</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>horse</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pig</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>woodchuck</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>possum</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tammar</td>
<td>mstksmdrv elaeelvkk agggqqgssrc lcls1fshll vaGatl1fCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>---------</td>
<td>--------</td>
<td>------</td>
<td>--------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>----------</td>
<td>--------</td>
<td>------</td>
<td>------</td>
<td>----------</td>
</tr>
</tbody>
</table>

-----------

<table>
<thead>
<tr>
<th></th>
<th>cow</th>
<th>goat</th>
<th>human</th>
<th>cat</th>
<th>dog</th>
<th>horse</th>
<th>pig</th>
<th>mouse</th>
<th>woodchuck</th>
<th>rabbit</th>
<th>possum</th>
<th>tammar</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>201</td>
<td>238</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>goat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>horse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>woodchuck</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>possum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tammar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>--GVF-L---</td>
<td>D-L---E-N-P</td>
<td>--LD--E-GQ--</td>
<td>--YFG-IAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Figure 2.1: Consensus Alignment of TNF-alpha Amino Acid Sequences.
Representative sequences from selected animal species were compared and the regions of greatest similarity were chosen as forward and reverse primer sites (shaded). Amino acids between 50 and 150 have been omitted in this figure for brevity (indicated by dashed blue line). Genbank accession numbers: cow (Q06599), goat (P13296), human (P01375), cat (P19101), dog (P51742), horse (P29553), pig (P23563), mouse (P06804), woodchuck (O35734), rabbit (P04924), possum (P79374), tammar (O77764).
Figure 2.2: Consensus Alignment of TNF-alpha Nucleotide Sequences. After selection of the region of greatest similarity in the amino acid alignment, nucleotide sequences within this region were inspected and primers designed to include the nucleotides showing the greatest consensus. Tammar sequence was inserted into the primer for areas where similarity was low. Forward and reverse primer sites are shown here (shaded). Nucleotides between 400 and 800 have been omitted in this figure for brevity (indicated by dashed blue line). Accession numbers: human (X01394), horse (JQ1344), pig (X54859), sheep (P23383), possum (AF016102), tammar (AF055915).
Figure 2.3: Consensus Alignment of IL-1β Amino Acid Sequences.

Representative sequences from selected animal species were compared and the regions of greatest similarity were chosen as forward and reverse primer sites (shaded). Amino acids between 150 and 200 have been omitted in this figure for brevity (indicated by dashed blue line). Genbank accession numbers: sheep (P21621), cow (P09428), deer (P51745), pig (P26889), cat (P41687), horse (Q28386), mouse (P10749), rat (Q63264), monkey (P79182), human (P01584), rabbit (P14628), possum (AAD21871).
**Figure 2.4: Consensus Alignment of IL-1β Nucleotide Sequences.** After selection of the region of greatest similarity in the amino acid alignment, nucleotide sequences within this region were inspected and primers designed to include the nucleotides showing the greatest consensus. Possum sequence was inserted into the primer for areas where similarity was low. Forward and reverse primer sites are shown here (shaded). Nucleotides between 450 and 800 have been omitted in this figure for brevity (indicated by dashed blue line).

Accession numbers: mouse (M15131), cow (M35589), horse (D42147), rabbit (D21835), human (X02532), monkey (D63353), possum (AF071539), chicken (Y15006).
Figure 2.5: Consensus Alignment of IL-10 Amino Acid Sequences.
Representative sequences from selected animal species were compared and the regions of greatest similarity were chosen as forward and reverse primer sites (shaded). Genbank accession numbers: gerbil (AAA65677), mouse (P18893), cat (P55029), dog (P48411), human (P22301), horse (Q28374), cow (P43480), sheep (Q29408), deer (P51746), whale (O4663), pig (Q29055), possum (O97798).
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gerbil</td>
<td>hamster</td>
<td>cow</td>
<td>horse</td>
</tr>
<tr>
<td></td>
<td>TACTGGGT</td>
<td>TACTGGGT</td>
<td>TACTGGGAT</td>
<td>TACTGGGAT</td>
</tr>
<tr>
<td></td>
<td>CCCAGCTT</td>
<td>CCCAGCTT</td>
<td>TCCAGGCTT</td>
<td>TCCAGGCTT</td>
</tr>
<tr>
<td></td>
<td>aTCaGAaATG</td>
<td>aTCaGAaATG</td>
<td>gTCGGAaATG</td>
<td>gTCGGAaATG</td>
</tr>
<tr>
<td></td>
<td>ATccAGTTTT</td>
<td>ATccAGTTTT</td>
<td>ATccAGTTTT</td>
<td>ATccAGTTTT</td>
</tr>
<tr>
<td></td>
<td>ACCTGGTaaGA</td>
<td>ACCTGGTaaGA</td>
<td>ACCTGGTaaGA</td>
<td>ACCTGGTaaGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pig</td>
<td>possum</td>
<td>Consensus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TACTGGGT</td>
<td>TACTGGGCT</td>
<td>TACT---TT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCCAGCTT</td>
<td>CCCAGgCTT</td>
<td>GCCA--CCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gTCGGAATG</td>
<td>ATtaAGTTTT</td>
<td>-TC- GA- ATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATccAGTTTT</td>
<td>ATccAGTTTT</td>
<td>ATccAGTTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACCTGGaAGA</td>
<td>ACCTGGaAGA</td>
<td>ACCTGGaAGA</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.6: Consensus Alignment of IL-10 Nucleotide Sequences.** After selection of the region of greatest similarity in the amino acid alignment, nucleotide sequences within this region were inspected and primers designed to include the nucleotides showing the greatest consensus. Possum sequence was inserted into the primer for areas where similarity was low. Forward and reverse primer sites are shown here (shaded). Nucleotides between 350 and 500 have been omitted in this figure for brevity (indicated by dashed blue line).

Accession numbers: gerbil (L37781), hamster (AF046210), cow (U00799), horse (U38200), pig (L20001), possum (AF026277).
SECTION II

The Tammar Wallaby

(*Macropus eugenii*)

‘A Model Marsupial Species’
Introduction

The Use of Model Species

The establishment of the core principles of eutherian immunology was assisted by the use of murine animal model systems that helped to elucidate many immunological mechanisms in other vertebrate species (Roitt, 1991). However, as the knowledge base grew and the breadth of the experimental base expanded, differences in the immune responses of unrelated animals became evident (Farrar and Murphy, 2000). The limitations of these experimental models created the need for newer, more closely related model species that accurately reflected the immunobiological responses of target species.

The concept of the use of marsupials as model species is not new and apart from the phylogenetic considerations mentioned in Chapter One, marsupials are used as model animals for a variety of eutherian biological studies. These include, but are not limited to, immunological studies (Brozek and Ley, 1991; Montali et al., 1998), lactational, biomedical and genetic studies (Tyndale-Biscoe and Janssens, 1988; Hinds et al., 1990) and reproductive studies (Mate et al., 1998; Nave et al., 2000).

In addition to their applications to eutherian studies, marsupial models have also been used to investigate immunobiological responses within the Marsupialia. In these studies, responses of the Australian brushtail possum (Moriarty and Thomas, 1986) and the American opossums, M. domestica (Stone et al., 1996) and D. virginiana (Taylor and Burrell, 1968), have been recorded. However, these animals represent only a very small proportion of marsupial species. Therefore, assumptions made from limited testing of these relatively few animals must be interpreted with care. The current study required an animal model from within the family Macropodidae to allow optimisation of immunological techniques for application to other macropod species. The tammar wallaby was selected as this model animal.

The Tammar Wallaby

The tammar wallaby was selected for use as a model Macropodidae species for a number of reasons. M. eugenii, like a number of small macropods, has been affected
by mycobacterial disease (Peet and Dickson, 1982) and this is of relevance since other species in the present study are believed to be susceptible to this intracellular pathogen (Phelan, 1996). However, unlike most other small macropods, this animal breeds well in captivity (Renfree, 1981a) and is available for research studies since there are a number of experimental colonies established within Australia. Tammar wallabies enjoy a high research profile due to their use in reproductive studies and, of particular relevance to the current work, are classified in the same family as the Rufous Hare-wallaby and the potoroid species (Kirsch et al., 1997). Thus, characterisation of the cellular immune responses in these animals provides a benchmark for the assessment of immunological mechanisms available to other macropod species vulnerable to mycobacterioses, such as the *Potoroidae*.

This section describes the isolation, characterisation and *in vitro* functional capacity of cells that mediate specific immune responses, the lymphocytes, and those primarily responsible for innate immune responses, the phagocytes. Collectively, this data provides the basis for comparison of similar responses measured in the Rufous hare-wallaby and potoroid species detailed in Section III of this study.
CHAPTER THREE
Identification and Enumeration of Blood Leukocytes

3.1 Introduction
Circulating leukocytes play a pivotal role in the regulation of the immune response and functional studies of these cells are often undertaken in vitro because of the relatively non-invasive nature of this form of testing and the accessibility of peripheral blood cell samples. However, before meaningful functional studies can be undertaken on individual cell populations, it is essential that their identity is determined and that reliable techniques are established for their isolation. Whilst a number of marsupial studies have classified white blood cells at the light microscope level during studies of total cell numbers or differential cell counts (see Table 3.1), this is the first study to describe the detailed morphology of tammar wallaby peripheral blood leukocytes using both light and electron microscopy.

Lymphocytes can be distinguished from other leukocytes by their morphology at the light microscope level, but they cannot easily be differentiated from each other using these same physical characteristics. It is possible however, to identify lymphocyte sub-populations using the technique of immunocytochemistry. In this procedure, the presence of surface markers unique to each of the cell types is detected using an antibody that recognises these surface marker antigens. Standardisation of the nomenclature for these surface proteins has resulted in the assignment of cluster of differentiation (CD) designations for each surface antigen (Burkitt et al., 1993).

In the present study, antibodies raised to eutherian T cell surface antigens, CD3 and CD5, were used to characterise peripheral blood marsupial lymphocytes. Antibodies raised to these cell markers have been used to identify T cells in the peripheral blood of a range of eutherian species including cows, dogs, humans, mice, sheep and pigs (Levkutova and Revajova, 1997; Appleyard and Wilkie, 1998). Whilst the distribution of different lymphocyte populations within marsupial lymphoid tissue beds has been demonstrated using these and other antibodies (Coutinho et al., 1995;
Hemsley et al., 1995; Baker et al., 1999), the distribution of different lymphocyte populations within peripheral blood leukocytes has only been reported for the koala (Wilkinson et al., 1995). This chapter describes the morphology and incidence of the five main leukocyte populations of the tammar wallaby. The development and application of an immunocytochemical procedure that facilitated the differentiation of subpopulations of circulating lymphocytes is also described.

3.2 Results

3.2.1 Separation of Individual Cell Populations
Tammar wallaby leukocyte populations were successfully separated into lymphocyte, monocyte-derived adherent cells and granulocytes. Results relating to the optimum cell separation conditions and the efficiency of these techniques are presented in Chapters 4 and 5, since the method of cell isolation was related to the functional capacity of the different cell populations described therein.

3.2.2 Cell Classification and Enumeration

3.2.2.1 Morphology of Tammar Wallaby Peripheral Blood Cells
In blood smears prepared and stained according to Method 2.3.3.1, erythrocytes appeared as anucleated biconcave discs that measured 6.0-8.0 μm in diameter (Figure 3.1a-i). Nucleated red blood cells (nRBC) were routinely found in blood smears but generally did not exceed 1% of the total differential count. However, on one occasion, a differential count of 50% nRBC was obtained for an animal that was injured and later found to have suffered from internal bleeding. Howell-Jolly bodies (small nuclear remnants in RBCs) and Heinz bodies (focal areas of oxidised haemoglobin) (Haen, 1995), were occasionally seen in tammar red blood cells. Platelets measuring between 2.0 and 4.0μm were routinely observed in blood smears (Figure 3.1g and i) and were occasionally present as large aggregates.
Figure 3.1: Peripheral Blood Leukocytes of the Tammar Wallaby.
Peripher al blood smears were stained with Diff Quik differential stain after fixation in
methanol. In the first row, (a) – (c) are small, medium and large lymphocytes
respectively. Note the slightly indented nucleus in (a) (arrow). Two monocytes (d)
and (e) and an eosinophil (f) are in the second row. A rarely seen basophil (g), an
unidentified cell commonly seen in blood films (h) and a mature neutrophil (i) are
shown in the bottom row. Biconcave erythrocytes surround all leukocyte types and
platelets of various sizes are clearly visible in figures (g) and (i). Scale bar = 10µm.
3.2.2.1.1 Lymphocytes

Lymphocytes were typically round in appearance and possessed a single, unilobed nucleus that was eccentrically located (Figures 3.1a-c). A small number of lymphocytes, usually the small to medium sized cells, contained a nucleus that was slightly indented (Figure 3.1a). The nuclear chromatin was generally dense and clumped, staining a deep crimson to purple colour with Diff Quik and Giemsa stains. The cytoplasm of these cells stained a variable blue colour, indicative of a basophilic nature, and sometimes contained azurophilic granules (not shown). This blue staining was lighter in the larger cells. Lymphocytes were characterised by a high nuclear to cytoplasm ratio that decreased as cells increased in size. The mean lymphocyte cell diameter was 9.6 ± 2.6μm (range 5.5μm to 17.5μm) but as is evident from the large standard deviation, these cells were not of uniform size. Therefore, in this study, lymphocytes were classified into three size classes; small (diameter 5-9μm), medium (9-15μm) and large (>15μm). In most blood samples, the small and medium cells were the more frequently observed lymphocytes. Large lymphocytes were similar in size to monocytes and were distinguished from them by the darker blue colour of their cytoplasm, the shape of the nuclei and the characteristics of the nuclear chromatin.

A minority of leukocytes possessed distinct perinuclear pale-staining regions and an eccentrically placed nucleus. These characteristics are exhibited by activated B cells (plasma cells) of other mammals (Zucker-Franklin et al., 1981) and were thus classified as lymphocytes in this study. Binucleated cells possessing the staining characteristics of lymphocytes were regularly seen in blood smears and were also recorded as lymphocytes.

At the ultrastructural level, lymphocytes possessed a large nucleus that sometimes contained one or two nucleoli (Figures 3.2 and 3.3b). These cells were again characterised by a high nuclear to cytoplasm ratio. The nucleus was enveloped by a thin rim of cytoplasm and contained dense heterochromatin with regions of lighter staining euchromatin (the synthetically active form of chromatin) clearly evident. In most cases, the euchromatin was continuous with the nuclear pores (Figure 3.3a and 3.4a). Some cells were classified as lymphoblasts since the nucleus almost filled the entire cytoplasm and was largely composed of euchromatin (Figure 3.3b). A small number of mitochondria, ribosomes and lysosomes were visible in the scant
cytoplasm of most lymphocytes (Figure 3.4). Golgi apparati consisting of isolated profiles of smooth and rough endoplasmic reticulum and membrane-enclosed vesicles were identified in these cells (Figure 3.4). Vacuoles, assumed to be artefacts of lipid droplets, were also occasionally observed. The cell perimeter appeared smooth with occasional short and isolated microvilli. No centrioles were visible in any of the cells examined in this study.

Plasma cells were readily identified by the presence of the cisternae of rough endoplasmic reticulum that filled the cytoplasm and the prominent perinuclear Golgi apparatus (Figure 3.4).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Mean (μm) ± SD</th>
<th>Range (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>86</td>
<td>9.0 ± 2.6</td>
<td>5.5 – 17.5</td>
</tr>
<tr>
<td>Monocyte</td>
<td>16</td>
<td>16.0 ± 3.2</td>
<td>12.4 – 23.4</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>11</td>
<td>12.4 ± 2.6</td>
<td>8.2 – 16.4</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>10</td>
<td>13.5 ± 2.1</td>
<td>10.5 – 16.8</td>
</tr>
<tr>
<td>Basophil</td>
<td>2</td>
<td>14.0 ± 0.6</td>
<td>13.6 – 14.4</td>
</tr>
</tbody>
</table>

Table 3.1: Diameters of Tammar Wallaby Peripheral Blood Leukocytes. Cell diameters were measured in peripheral blood smears using an eyepiece micrometer at 1000x magnification after slides were stained with Diff Quik differential stain.

3.2.2.1.2 Monocytes

When treated with Diff Quik differential stain, the cytoplasm of monocytes stained a light blue to grey colour and contained a light crimson to lilac-coloured nucleus (Figure 3.1d and e). In general, monocytes were larger than lymphocytes (Table 3.1) and possessed a single nucleus that was typically kidney-shaped (Figure 3.1d). The nucleus of some cells was almost spherical and difficult to classify. In these cases, monocytes were distinguished from large lymphocytes by the threadlike quality of the nuclear chromatin, the differences in staining intensity (monocytes generally take on a lighter staining pallor than lymphocytes) and the ratio of nucleus to cytoplasm (lymphocytes generally contain less cytoplasm for the same cell size). Monocyte cell diameters ranged between 12.4 and 23.4μm. The cytoplasm of these cells was occasionally vacuolated but did not contain visible granules.
At the ultrastructural level, the cytoplasm of monocytes contained very few granules. Specifically, lysosomal granules were not apparent in any of these cell types inspected in this study. However, these cells contained a range of vesicle forms, abundant ribosomes and prominent RER especially along the periphery of the cell (Figure 3.5). In section, monocytes appeared bilobed which is consistent with the kidney-shaped nucleus that characterises these cell forms (Figure 3.5a). Microvillus-like projections were clearly visible along the outline of the cell and villus-enclosed phagocytic structures were occasionally observed (Figure 3.5b).
Figure 3.2: Ultrastructure of Tammar Wallaby Mononuclear Cells. The cell at the centre of the field lacks distinguishing features and may be either a medium-sized lymphocyte or small monocyte. In the lower left of field a plasmacytoid lymphocyte has extensive rough endoplasmic reticulum (RER) in the cytoplasm and prominent nucleoli (white arrow) within the nucleus (nu). A cell containing large membrane enclosed vacuoles (black arrow), prominent RER, mitochondria (m) and extensive membrane projections is visible on the far right of picture. Magnification x7800.
Figure 3.3: Ultrastructure of Tammar Wallaby Peripheral Blood Lymphocytes. Figure (a) is a micrograph showing the typical morphology of resting lymphocytes with the nuclei (n) occupying most of the cell volume and composed principally of electron-dense heterochromatin (h). Note the lighter-stained euchromatin (e) that is continuous with the nuclear pores (*). Magnification x 13 200. Figure (b) is a group of more active lymphocytes. The largest cell is presumably a lymphoblast (B) with abundant light-staining euchromatin. The cell at the top left of field also contains more euchromatin than the lymphocytes in figure (a). Few mitochondria are present in these cells (black arrows) but a prominent nucleolus can be seen in the nucleus of the cell in the lower left of field (white arrow). Magnification x10 400.
Figure 3.4: Two Different Types of Tammar Wallaby Lymphocyte. Figure (a) has few distinguishing features apart from the large nucleus (n_u) to cytoplasm (c) ratio and general morphology. The cytoplasm of this cell is filled with many darkly stained free ribosomes. Mitochondria (m) are visible at the top left of the nucleus. A membrane-enclosed cytoplasmic vesicle (v) is also clearly visible. Magnification x 17,800. Figure (b) is a peripheral blood lymphocyte with all the distinguishing features of a plasma cell. Clearly visible are the (G) Golgi apparatus, extensive rough endoplasmic reticulum (RER) and mitochondria (m). Note the prominent nucleolus (n) within the nucleus (n_u). Magnification x 23,000.
Figure 3.5: Tammar Wallaby Peripheral Blood Mononuclear Cells.

Figure (a) has a lymphocyte (L) at the top of field. The monocyte (M) appears binucleated but this is most likely the effect of sectioning through the characteristic horseshoe nucleus (n). Magnification x10 400. Figure (b) is a monocyte with prominent nucleolus (n) and an obvious group of mitochondria (m). The cytoplasm also contains many membrane-enclosed vesicles (v). Note the newly formed phagocytic vacuole at left of picture (arrow). Monocytes in both micrographs possess characteristic cytoplasmic microvilli extending from the cell surface. Magnification x 13 200.
3.2.2.1.3 Granulocytes

Granulocytes were easily identified at the light and electron microscope level by their unique staining characteristics and the presence of different types of granules. Neutrophils and eosinophils were common but basophils were rarely detected in prepared blood smears and were not present in samples prepared for electron microscopy.

Neutrophils

Neutrophils were the most frequently observed granulocyte in tammar wallaby blood films. The majority of neutrophils were the mature multilobed variety with strands of nucleoplasam visible within a polymorphonuclear structure (Figure 3.1i). Cells possessing the morphology of immature ‘left shift’ (Haen, 1995) neutrophils were present in some samples. These cells were more eosinophilic in character than large lymphocytes and monocytes and possessed large nuclei with less visible cytoplasm than monocytes. Occasionally, band cell forms (immature neutrophils) were visible. Mature neutrophils were distinguished from other cells by the morphology of their nuclei. The nucleus of most neutrophils stained a strong purple colour with Diff Quik and Giemsa stains and the cytoplasm either stained faintly pink to lilac or appeared to be transparent so that cytoplasmic margins were difficult to define. Where granules were visible, they appeared as discrete, light purple stained structures that were evenly distributed throughout the cytoplasm (faintly visible in Figure 3.1i).

A number of nuclear morphologies were encountered in blood films. An unusual form of immature cell, an annular neutrophil, was seen in two animals but were not detected in any other samples. The shape of some nuclei was tube-like with areas that were pinched-in rather than discretely separated by filaments. Others possessed up to four large clumps of nuclear material that appeared to overlap or fold up upon one another. The majority of cells possessed nuclear lobes that were joined by thin filaments of nucleoplasam as previously described. The number of lobes routinely identified in these samples varied from three to seven.

Granules and cytoplasmic organelles were more clearly distinguished in electron micrographs. The heterogeneity of granules in tammar wallaby neutrophils is apparent in Figures 3.6 and 3.7. In this study, granule types were classified on the
basis of morphology and there appeared to be at least three different forms of these cell inclusions. Larger granules measured approximately 0.4μm (longest diameter) and were electron dense in nature. Intermediate sized pleomorphic granules possessed a range of electron densities and some were almost indistinguishable from the cytoplasm itself. Dumbbell-shaped granules were also present in this group (Figure 3.7). Smaller, more distinctly rounded granules of less than 90nm in diameter varied in electron density and were also scattered throughout the cytoplasm. Whilst the plane of sectioning made quantitation of granule types and numbers problematic, it was evident that the intermediate and small granules were the most abundant forms present in neutrophils. A number of all types of granules possessed a clear region within the electron dense intra-granular region (see figure 3.6) which suggested the presence of a chemical core.

The multilobed structure of neutrophil nuclei was clearly evident in transmission electron micrographs (TEMs). The cell cytoplasm contained very sparse mitochondria that were not visible in all cells. Small profiles of RER were visible in the cytoplasm of some neutrophils but most did not appear to contain these structures. Similarly, Golgi apparatus was visible in some cells (Figure 3.6) but these structures were generally less extensive than in lymphocytes and were not frequently observed.

Eosinophils

Eosinophils were present in most, but not all samples of peripheral blood analysed in this study. When inspected using light microscopy after differential staining, these cells were generally larger than neutrophils (Table 3.1) and contained intensely pink to orange-stained large granules that filled the entire cytoplasm (Figure 3.11). The nuclei were either singular or bilobed and stained a sky-blue colour with Giemsa stain and a lilac to crimson colour with Diff Quik stain. All eosinophils inspected in this study were of this form with the exception of two incidences where an annular eosinophil was identified (animal 209) and in a different animal, an eosinophilic band cell (animal 11:722CFT) was observed.

At the ultrastructural level, eosinophil granules contained an obvious crystalloid structure that characterised these cells (Figure 3.8). Cytoplasmic vesicles were absent
but small Golgi Apparatus, few mitochondria, ribosomes and small profiles of RER were clearly visible.

Granules were typically round to elliptical in shape, with some appearing almost triangular (Figure 3.8a). Granule sizes varied from 0.18 – 0.82μm in width and 0.31 – 1.33μm in length. Granules were membrane-bound with an electron dense matrix and pale staining crystallloid core. This structure did not fill the entire volume of the granule and appeared to be composed of a number of discrete, striated crystals that ranged in width from 14.3 – 36.0nm and were of variable length, with most extending the length of the granule (Figure 3.8b).

Unidentified cells that possessed morphology similar to that of Figure 3.1h were tentatively identified as granulocytic blast cells. These cells possessed an eosinophilic, stringy cytoplasm, had no clearly defined nucleus, and were fragile and fragmented easily during the preparation of blood smears. When observed in an undamaged state, these cells measured between 15 and 30μm on the longest diameter and varied in shape from oval to amorphous. These cells were found to be phagocytic in later analyses (see 4.2.2) and were generally found in samples that also contained binucleated cell forms.

Basophils

Basophils were the least frequently encountered granulocyte. When stained with Diff Quik, the cytoplasms of these cells stained a very pale blue or were transparent and contained numerous fine basophilic granules (Figure 3.1). The nuclei were U-shaped or ovoid and were also covered with granules. With the exception of one cell that contained a large, euchromatic nucleus and cytoplasmic granules that did not appear to contain crystallloid inclusions similar to those identified in eosinophils (Figure 3.9), no basophils were detected in peripheral blood leukocyte samples inspected using electron microscopy.
Figure 3.6: Ultrastructure of Mature Tammar Wallaby Granulocytes. This electron micrograph shows the two main types of granulocytes found in the peripheral blood of the tammar wallaby. Neutrophils (N) possess multilobed nuclei and numerous small granules in the cytoplasm. Note the lysosomal granule indicated by the solid white arrow. Eosinophils (E) possess much larger granules that contain crystalline inclusions. A small Golgi Apparatus (G) is visible in the polymorphonuclear cell on the top right of picture. Nuclei (nu) are clearly visible in both cell types.
Figure 3.7: Ultrastructure of a Neutrophilic Granulocyte. The multilobed nucleus (nu) characteristic of this cell type is clearly visible in this micrograph. The cytoplasm is scattered with granules of various shapes, sizes and electron densities. Note the dumbbell-shaped granules (short arrows) and the large primary (p) and smaller secondary (s) granules.
Figure 3.8: Ultrastructure of Tammar Wallaby Peripheral Blood Eosinophilic Granulocytes. Figure (a) is a micrograph of a section through the cell where no nucleus is apparent. The cytoplasm is filled with numerous large granules that contain a visible crystalline structure. Figure (b) is a higher magnification of eosinophilic granules clearly demonstrating the fine, crystalloid inclusions (arrows).
Figure 3.9: Ultrastructure of Tammar Wallaby Granulocytes. Two eosinophils are visible at the top of field. (E) is a mature eosinophil with a visible nucleus (n_u) at the top of the cell and typical large granules. (Eb) has abundant euchromatin in the nucleus and may be a basophil since it has large, dense granules that do not appear to contain crystalloid inclusions. The appearance of the nuclear arrangement in neutrophils (N) is affected by the plane of section. Both neutrophils contain many cytoplasmic granules.
3.2.2.2 Enumeration of Tammar Wallaby Leukocytes

Quantitation of the relative and total cell numbers present in the periperal blood of tammar wallabies was carried out by direct counting of cells in whole blood (Method 2.3.3.3.1) and by performing a differential count of the leukocyte populations in blood smears (Method 2.3.3.4).

3.2.2.2.1 Total Leukocyte Counts

Tammar wallaby total leukocyte counts ranged from $2.2 \times 10^6$ cells/mL to $10.7 \times 10^6$ cells/mL. Results for each animal are presented as the mean cell count per animal averaged over the entire study period (Table 3.2).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mean Cell Number ± (SD) (x10^6 cells/mL)</th>
<th>Sample number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400 $^*$</td>
<td>3.6 ± 0.5</td>
<td>4</td>
</tr>
<tr>
<td>1515 $^*$ (juvenile)</td>
<td>8.6</td>
<td>1</td>
</tr>
<tr>
<td>1583 $^*$ (juvenile)</td>
<td>8.5 ± 1.2</td>
<td>2</td>
</tr>
<tr>
<td>1612 $^*$</td>
<td>3.6 ± 0.1</td>
<td>2</td>
</tr>
<tr>
<td>1652 $^*$</td>
<td>4.9 ± 0.7</td>
<td>2</td>
</tr>
<tr>
<td>1657 $^*$</td>
<td>6.2 ± 0.9</td>
<td>2</td>
</tr>
<tr>
<td>1844 $^*$</td>
<td>4.8 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>1958 $^*$</td>
<td>5.5</td>
<td>1</td>
</tr>
<tr>
<td>1994 $^*$</td>
<td>8.3 ± 1.4</td>
<td>3</td>
</tr>
<tr>
<td>8021 $^*$</td>
<td>3.8 ± 1.1</td>
<td>3</td>
</tr>
<tr>
<td>16F4CB2T $^*$</td>
<td>3.2 ± 0.6</td>
<td>6</td>
</tr>
<tr>
<td>16F22C8T $^*$</td>
<td>9.6 ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>13788BAT $^*$</td>
<td>6.0 ± 1.1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.2: Tammar Wallaby Peripheral Blood Total Leukocyte Counts.

Haemocytometer counts of the total number of leukocytes in whole blood diluted 1/10 with Turk's counting solution were obtained under 400x magnification.

3.2.2.2.2 Differential Cell Counts

Differential cell counts for individual animals obtained during this study are presented in Tables 3.3 and 3.4.
<table>
<thead>
<tr>
<th>Animal</th>
<th>n</th>
<th>% Lymphocytes (L) mean ± SD</th>
<th>% Neutrophils (N) mean ± SD</th>
<th>L:N %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1008</td>
<td>5</td>
<td>52.6 ± 11.8</td>
<td>43.1 ± 11.1</td>
<td>55:45</td>
</tr>
<tr>
<td>1400</td>
<td>10</td>
<td>44.5 ± 10.8</td>
<td>48.1 ± 9.3</td>
<td>52:48</td>
</tr>
<tr>
<td>1612</td>
<td>13</td>
<td>64.1 ± 6.6</td>
<td>32.2 ± 7.2</td>
<td>67:33</td>
</tr>
<tr>
<td>1652</td>
<td>12</td>
<td>52.3 ± 11.2</td>
<td>42.9 ± 10.8</td>
<td>55:45</td>
</tr>
<tr>
<td>1657</td>
<td>11</td>
<td>71.0 ± 9.2</td>
<td>25.8 ± 8.0</td>
<td>73:27</td>
</tr>
<tr>
<td>1844</td>
<td>13</td>
<td>48.6 ± 11.3</td>
<td>45.9 ± 10.9</td>
<td>51:49</td>
</tr>
<tr>
<td>1958</td>
<td>2</td>
<td>75.6 ± 9.0</td>
<td>15.3 ± 7.1</td>
<td>83:17</td>
</tr>
<tr>
<td>1994</td>
<td>7</td>
<td>80.0 ± 3.2</td>
<td>15.7 ± 2.4</td>
<td>84:16</td>
</tr>
<tr>
<td>202</td>
<td>2</td>
<td>62.4 ± 11.4</td>
<td>30.0 ± 13.6</td>
<td>68:32</td>
</tr>
<tr>
<td>209</td>
<td>5</td>
<td>54.6 ± 11.0</td>
<td>38.8 ± 12.1</td>
<td>58:42</td>
</tr>
<tr>
<td>6460</td>
<td>4</td>
<td>64.8 ± 14.5</td>
<td>30.2 ± 11.9</td>
<td>68:32</td>
</tr>
<tr>
<td>6693</td>
<td>7</td>
<td>75.9 ± 5.2</td>
<td>17.3 ± 5.9</td>
<td>81:19</td>
</tr>
<tr>
<td>8021</td>
<td>17</td>
<td>60.7 ± 10.5</td>
<td>34.1 ± 10.0</td>
<td>64:36</td>
</tr>
<tr>
<td>12F3C0D1</td>
<td>10</td>
<td>71.7 ± 9.2</td>
<td>25.1 ± 8.3</td>
<td>74:26</td>
</tr>
<tr>
<td>12F5O941</td>
<td>2</td>
<td>38.2 ± 8.6</td>
<td>55.7 ± 7.2</td>
<td>41:59</td>
</tr>
<tr>
<td>13788BAT</td>
<td>6</td>
<td>78.0 ± 8.4</td>
<td>19.4 ± 7.9</td>
<td>80:20</td>
</tr>
<tr>
<td>1F722CF1</td>
<td>11</td>
<td>62.2 ± 8.2</td>
<td>31.7 ± 6.3</td>
<td>66:34</td>
</tr>
<tr>
<td>1F4CB21</td>
<td>4</td>
<td>70.4 ± 3.3</td>
<td>26.2 ± 3.4</td>
<td>73:27</td>
</tr>
</tbody>
</table>

**Table 3.3: Ratio of Lymphocytes to Neutrophils in Tammar Wallaby Peripheral Blood.** The percentage of Lymphocytes (L) and Neutrophils (N) was obtained from differential cell counts and are presented as mean ± one standard deviation. The relationship between them was expressed as percentage L:N ratios and was calculated using data over the entire period of the study. \( n \) = number of different sampling events.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Monocytes (mean% ± SD)</th>
<th>Eosinophils (mean% ± SD)</th>
<th>Basophils (incidence and mean%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1008</td>
<td>2.3 ± 1.5</td>
<td>1.9 ± 1.2</td>
<td>0/5</td>
</tr>
<tr>
<td>1400</td>
<td>4.0 ± 2.0</td>
<td>3.2 ± 1.5</td>
<td>2/10 at 0.8 &amp; 1.0%</td>
</tr>
<tr>
<td>1612</td>
<td>2.3 ± 1.3</td>
<td>1.6 ± 0.7</td>
<td>1/13 at 0.4%</td>
</tr>
<tr>
<td>1652</td>
<td>2.3 ± 1.1</td>
<td>2.5 ± 2.1</td>
<td>1/12 at 0.4%</td>
</tr>
<tr>
<td>1657</td>
<td>2.2 ± 2.0</td>
<td>0.9 ± 0.9</td>
<td>0/11</td>
</tr>
<tr>
<td>1844</td>
<td>3.3 ± 1.5</td>
<td>1.7 ± 1.0</td>
<td>2/13 at 0.7%</td>
</tr>
<tr>
<td>1994</td>
<td>2.4 ± 1.9</td>
<td>1.9 ± 1.7</td>
<td>1/7 at 0.8%</td>
</tr>
<tr>
<td>209</td>
<td>3.7 ± 1.2</td>
<td>2.9 ± 2.0</td>
<td>0/5</td>
</tr>
<tr>
<td>12F3CODT</td>
<td>1.6 ± 1.2</td>
<td>1.7 ± 1.3</td>
<td>1/10 at 0.4%</td>
</tr>
<tr>
<td>13788BAT</td>
<td>1.0 ± 0.8</td>
<td>1.5 ± 1.4</td>
<td>0/6</td>
</tr>
<tr>
<td>1F722CFT</td>
<td>2.9 ± 1.7</td>
<td>3.3 ± 2.2</td>
<td>0/11</td>
</tr>
</tbody>
</table>

**Table 3.4: Tammar Wallaby Monocyte, Eosinophil and Basophil Differential Counts.** Results recorded here are for animals where five or more samples were analysed. Both the incidence and mean % of basophils are reported. Incidence refers to the number of blood smears where basophils were detected out of the total number analysed for that particular animal. Where basophils were detected, their mean % of the differential count is also reported.

**Lymphocytes and Neutrophils**

In most blood samples, lymphocytes were the most commonly observed leukocyte (Table 3.3). Whilst medium and small lymphocytes formed the majority of these cells, large lymphocytes were sometimes present in greater numbers and were usually detected when the unidentified cells in Figure 3.1(h) were also apparent. The second most commonly observed cell was the neutrophil and the majority of these were of the mature, multinucleated variety. Cells identified as putative granulocytic blast cells were present in blood smears at levels not exceeding 3% of the differential count.

Lymphocyte to Neutrophil (L:N) ratios are presented as relative percentages in Table 3.3. For six animals followed during the course of this study, this relationship is illustrated graphically in Figure 3.10. In most cases, when data was analysed over the entire study period, L:N ratios were greater than 1. The notable exception to this was animal 12F5094T that died during the study. Figure 3.10 illustrates the within-animal and between-animal changes in lymphocyte and neutrophil counts that
occurred during the study period and demonstrates the lack of an obvious trend amongst the counts of different animals.

**Monocytes, Eosinophils and Basophils**

The results of differential counts obtained for monocytes, eosinophils and basophils are presented in Table 3.4. Monocytes were detected in all blood smears and constituted 2.7% ± 0.2% (mean ± standard error, SE) of the differential count. The mean eosinophil count was 2.2% ± 0.2% (SE) and whilst these cells were observed in the blood of all animals at different times within the study, they were not present at every sampling event. In contrast, basophils were not detected in the blood smears of 46% of the animals tested and were the least commonly observed leukocyte. Of the seven animals where basophils were recorded, only one or two smears per animal were positive for this cell type during the study period. Where these cells were detected, they were present at between 0.4 and 1.0% of the differential cell count.

Where information was available, absolute cell counts were calculated for the individual leukocyte populations from each animal (Table 3.5). This data was generated using the mean differential count and the mean total leukocyte count for each animal sampled over the entire study period. These calculations were only performed when five or more differential counts were available.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Lymphocytes $x10^6$ cells/mL</th>
<th>Neutrophils $x10^6$ cells/mL</th>
<th>Monocytes $x10^6$ cells/mL</th>
<th>Eosinophils $x10^6$ cells/mL</th>
<th>Basophils $x10^6$ cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400</td>
<td>1.60</td>
<td>1.73</td>
<td>0.14</td>
<td>0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>1612</td>
<td>2.31</td>
<td>1.16</td>
<td>0.08</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>1652</td>
<td>2.56</td>
<td>2.10</td>
<td>0.11</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>1657</td>
<td>4.40</td>
<td>1.60</td>
<td>0.14</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>1844</td>
<td>2.33</td>
<td>2.20</td>
<td>0.16</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>1994</td>
<td>6.64</td>
<td>1.30</td>
<td>0.20</td>
<td>0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>1E722CFT</td>
<td>1.99</td>
<td>1.01</td>
<td>0.09</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>13788BAT</td>
<td>4.68</td>
<td>1.16</td>
<td>0.06</td>
<td>0.09</td>
<td>0</td>
</tr>
<tr>
<td>12F3C PDT</td>
<td>6.88</td>
<td>2.41</td>
<td>0.15</td>
<td>0.16</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Table 3.5: Tammar Wallaby Absolute Leukocyte Counts.** Absolute cell counts were calculated for animals over the entire study period where 5 or more differential counts were available.
Figure 3.10: Neutrophil and Lymphocyte Trends of Six Tammar Wallabies.
Differential cell counts were performed on samples collected over the entire study period. Logarithmic trends are shown to demonstrate the relative changes in cell ratios over time.
3.2.2.3 Incidental Blood Findings

During routine preparation and screening of blood samples for analysis, a number of different organisms were observed in the peripheral blood of test animals. These were not investigated further due to their low incidence but are mentioned here for their potential value in future studies.

i) Flagellated organisms were visible in cell preparations of animal 1652 on 13/01/98. These were very small, measuring approximately 3μm when observed in culture. Also visible but in very small numbers, were larger structures that possessed a single waist-like constriction. These were motile and difficult to photograph for later identification. A leukocyte smear prepared from animal 12F5094T also contained a flagellated organism, visible in Figure 3.11a. When stained with Giemsa stain, this organism possessed an obvious nucleus, a single flagellum and two prominently stained areas in what appeared to be the anterior section of the organism.

ii) Figure 3.11(b) is a large cyst found in the purified mononuclear cell preparation of animal 1400. This structure is consistent in size and morphology with the unsporulated cysts of T. gondii.

iii) Structures resembling unsporulated coccidian oocysts were visible in Turk’s treated whole blood of tammar wallaby 1E722CFT (Figure 3.11c). These structures possessed between 3-5 transparent small circular regions contained within a spherical, sometimes amorphous body that measured between 10-20μm in diameter.
Figure 3.11: Organisms found in the Blood of Apparently Healthy Tammar Wallabies. Figure (a) is an organism found in a mononuclear leukocyte preparation of animal 12F5094T. After staining with Giemsa stain, some internal structure was visible. Note the flagellum and anterior spots (arrow). Figure (b) is a trypan blue stained unsporulated Toxoplasma gondi cyst found in the cell preparation of animal 1400. Structures resembling coccidian oocysts that contained obvious cell inclusions (arrow) were found in the blood of animal 1E722CFT (c). Scale bar 10μm.
3.2.2.4 Phenotyping of Leukocytes

Leukocyte populations were further characterised by detection of the peroxidase enzyme in phagocytic cell populations and by the immunolabelling of lymphocytes.

3.2.2.4.1 Peroxidase Activity

Peroxidase activity was assessed in granulocytes and monocytes according to Method 2.3.8.4.6. In fresh preparations of isolated mononuclear cells there was no evidence of a brown deposit indicative of peroxidase enzyme activity. However, when cells were cultured overnight and re-tested, a few large mononuclear cells stained brown within the cell cytoplasm (Figure 3.12a).

The presence of peroxidase was also investigated in granulocytes. Cells isolated from animals 1583 and 209 were peroxidase positive and cells from animal 12F5094T were negative in this test. In general, results from this assay were variable and were only positive when cells were stained on the day of preparation (see later discussion). Figure 3.12b illustrates peroxidase-positive granulocytes contained in a total leukocyte preparation from animal 1538. This preparation was used to minimise the possibility of enzyme degradation that may have arisen due to the extra processing steps involved in the isolation of purified granulocyte preparations.
**Figure 3.12: Cytochemical Localisation of Peroxidase in Tammar Wallaby Phagocytes.** Cell populations were treated with DAB in the presence of H$_2$O$_2$ and counterstained with haematoxylin to emphasise development of the brown product formed by the oxidation of DAB in the presence of the peroxidase enzyme. Figure a) PBMC-derived adherent cells. Peroxidase positive cells were identified in this preparation but were not representative of most of these cells. Figure b) Peroxidase positive granulocytes. Granulocytes are clearly visible in this total leukocyte preparation with multilobed nuclei and obvious brown staining. Scale bar 10μm.
3.2.2.4.2 Immunophenotyping

Using an immunocytochemical slide technique optimised during this study, a proportion of tammar wallaby peripheral blood lymphocytes stained positively for the pan T cell markers, CD3 and CD5 (Table 3.6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Animal</th>
<th>Antibody and Dilution</th>
<th>% Positively Stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-adherent ‘T’ cells</td>
<td>8021</td>
<td>mCD5 1/100</td>
<td>36</td>
</tr>
<tr>
<td>PBMC</td>
<td>12F3C0DT (a)</td>
<td>mCD5 1/100</td>
<td>40 ± 9</td>
</tr>
<tr>
<td>PBMC</td>
<td>12F3C0DT (b)</td>
<td>mCD5 1/100</td>
<td>34 ± 6</td>
</tr>
<tr>
<td>PBMC</td>
<td>1538</td>
<td>mCD5 1/100</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>PBMC</td>
<td>1612</td>
<td>pCD3 1/1000</td>
<td>62</td>
</tr>
</tbody>
</table>

Data is reported as positively stained cells per field of view (x 1000).
(a) 13 Nov 1998 (b) 29 May 2000

Table 3.6: Immunophenotype of Tammar Wallaby Peripheral Blood Lymphocytes

Optimisation Of Immunocytochemical Method

a. Fixation protocol

The method of cell fixation and sample storage conditions proved critical to the successful immunolabelling of isolated lymphocytes. In preliminary studies, a number of fixation methods were used in order to optimise cell adherence to slides and to preserve antigens. Freshly prepared, methanol-fixed tammar wallaby cells were successfully labelled using both anti-CD3 and anti-CD5 antibodies whilst methanol-fixed, aged samples prepared at the same time and then stored at room temperature for one to four weeks were negative when tested. A number of fixative solutions using various levels and combinations of 10% buffered formalin and methanol were subsequently trialled in an effort to preserve cell antigens. The optimum fixative solution was found to be 1:1 acetone/methanol applied at -20°C for 10mins, since cells treated with this protocol did not lift from the slides during
processing and retained their staining properties for up to 2 months after initial leukocyte application.

b. **Blocking of endogenous peroxidase and non-specific binding**
Hydrogen peroxide levels of 0.3 and 0.9% were inconsistent in blocking endogenous peroxidase activity and were abandoned for routine use. Concentrations of 1.5% hydrogen peroxide in both methanol and water were found to effectively block endogenous peroxidase when cells were exposed to these solutions for 45mins. Since 1.5% hydrogen peroxide in water was the most cost effective alternative, it was selected for use in further assays. Confirmation of the effectiveness of peroxidase blocking was achieved by analysing a total leukocyte preparation that contained peroxidase positive granulocytes. Immunostaining of total leukocyte smears using the anti-CD5 antibody resulted in positive labelling of lymphocytes and no visible brown reaction in cells with polymorphonuclear morphology (see Figure 3.13c).

A serum blocking solution of 10% horse serum in PBS was effective for most analyses. However, variations in background staining and some incidences of non-specific binding between assays resulted in whole serum being chosen as the optimum blocking reagent since it consistently prevented non-specific staining of cells.

b. **Immunolabelling**
Immunostaining and analysis of slides was most effective when small drops of cells were applied to slides in a localised area that could be easily identified during the study. This technique allowed small numbers of cells to be efficiently processed since they were easily located on the slide after immunostaining. Leukocyte smears were only effective when large numbers of cells were available that could be easily spread across the microscope slide.

In early studies, monoclonal anti-CD3 antibody (mCD3) was tested at 1/10 and 1/50 dilutions. This antibody did not stain as many cells as mCD5 so was not further investigated due to limited quantities of reagent available for further optimisation. Optimisation experiments for mCD5 were carried out using dilutions of 1/10, 1/50 and 1/100 of this antibody. The 1/100 dilution of mCD5 provided the most distinct labelling of lymphocytes without confounding background effects. The polyclonal
anti-CD3 antigen was tested at 1/50, 1/100 and 1/1000 dilutions. Both 1/50 and 1/100 dilutions of this antibody resulted in non-specific binding of test cells. A final dilution of 1/1000 and a reduced primary antibody application time of 15 minutes was found to be most effective for this antibody.

Staining of the CD5 antigen was very distinct and intense in the membrane and cytoplasm of lymphocytes and was optimal when used at a dilution of 1/100 when primary antibody was applied for 60 minutes. Staining of these cells was more intense in smaller diameter cells (Figure 3.13a) and lighter and more diffusely distributed in larger diameter cells (Figure 3.13b). The polyclonal CD3 antigens were more diffusely stained throughout the cell surface on all samples tested.
Figure 3.13: Tammar Wallaby Peripheral Blood Cells Stained with Anti-CD5 Antibody. Figures (a) and (b) are of lymphocyte smears prepared from density gradient separated blood preparations. Both CD5 positive and CD5 negative lymphocytes are visible in both pictures. Figure (c) is a total leukocyte preparation showing the effective blocking of endogenous peroxidase in granulocytic cells (arrows). CD5 positive lymphocytes are stained brown in these figures.
3.3 Discussion

The tammar wallaby possessed the five main types of leukocytes found in the peripheral blood of most mammals (Banks, 1981). At both the light and electron microscope level, these cells were similar to those of eutherian mammals, with the exception of an apparent lack of lysozyme granules in monocytes (Briggs et al., 1966; Syrén and Raeste, 1971). A number of haematological studies of marsupial species have classified peripheral blood leukocytes at the light microscopic level (Table 3.7 and later discussion) but only two record the ultrastructural properties of these cells. Canfield and Dickens (1982) investigated the morphology of koala blood cells at both the light and electron microscope level and concluded that these cells shared similar characteristics with other mammals apart from the appearance of the eosinophilic granulocytes. Mononuclear cells and neutrophils of the dasyurids S. crassicaudata and S. macroura are also reported to be similar to eutherian mammals but no basophils and only a single eosinophilic cell were observed in the blood of these species (Haynes and Skidmore, 1991). This is the first report that describes the leukocytes of the tammar wallaby at both the light microscope and ultrastructural level.

Lymphocytes

Tammar wallaby lymphocytes possessed lymphocyte morphology typical of eutherian cells, with very few organelles visible in a scant cytoplasm. The presence of small, medium and large lymphocytes that ranged in size from 5.5\(\mu\)m to 17.5\(\mu\)m is consistent with the findings in the possum (Barbour, 1972) and the Allied Rock-wallaby, Petrogale assimilis (Spencer and Speare, 1992) and those of eutherian mammals that rarely have a uniform cell diameter (Schalm et al., 1975; Banks, 1981). In the tammar wallaby, large lymphocytes approached the size of monocytes, which are usually the largest of the circulating blood cells (Burkitt et al., 1993). However, this situation is not unique amongst mammals, as horse monocytes are also of similar size to large lymphocytes (Banks, 1981).

Granulated lymphocytes were detected in the blood of some animals but these were not a common finding. Natural Killer (NK) cells appear as granular lymphocytes and it is therefore possible that these cells be present in small numbers in tammar wallaby blood. This proposition could not be tested in the current study since functional
analyses would be required to confirm this identification and these cells were not readily isolated from the remaining mononuclear cell population.

Lymphocytes resembling plasma cells were found in most of the tammar wallaby blood samples taken in this study. In humans, a proportion of plasma cells has a binucleated morphology, but are usually not observed in the peripheral circulation (Zucker-Franklin et al., 1981). They are, however, sometimes encountered in the blood of patients with viral infections. Binucleated lymphocytes have also been reported in sheep and deer (Schalm et al., 1975) and in another marsupial, the Allied Rock-wallaby, where up to 7% of circulating lymphocytes were of the binucleated variety (Spencer and Speare, 1992).

Monocytes may also be binucleated and in elephants, these cells can only be differentiated from lymphocytes by the presence of the peroxidase enzyme (Banks, 1981). Tammar wallaby monocytes did not reliably stain positively for peroxidase so this test could not be used to differentiate these cell types. Thus, the morphology and staining properties of the cells were used as the only discerning characteristics. All binucleated cell forms were thus counted as lymphocytes unless morphological and staining characteristics supported a monocytic lineage.

Absolute lymphocyte counts for the tammar wallaby were between $1.60 \times 10^6$ cells/mL and $6.88 \times 10^6$ cells/mL. This number was similar to that obtained for red-necked wallaby lymphocyte counts ($0.69 \times 10^6$ to $4.44 \times 10^6$ cells/mL; Hawkey et al., 1982) but differed in both range and maximum values from the Allied Rock-wallaby ($0.32 \times 10^6$ cells/mL to $15.14 \times 10^6$ cells/mL; Spencer and Speare, 1992).

**Monocytes**

Monocytes were detected in all tammar wallaby blood films prepared in this study and were morphologically similar at the light microscope level to those of eutherian mammals (Banks, 1981) and of other marsupials studied to date. However, unlike the monocytes of the Allied Rock-wallaby that possessed ‘dusky pink’ granules (Spencer and Speare, 1992), the cells of the tammar did not appear to contain visible cytoplasmic granules. At the ultrastructural level, lysosomal granules which are characteristic of these cells in eutherian mammals (Abbas et al., 1997), appeared to be
absent. These granules are usually identified as electron-dense structures that contain an opaque area when examined in transmission electron micrographs. Failure to detect these structures in the current study may be due to a processing artefact or the possibility of cell activation prior to processing. Bacterial products are known to activate monocytes (Baumann and Gauldie, 1994) and blood samples in the present study were sometimes contaminated with bacteria at the time of collection. It is also possible that the lack of lysosomal granules is a characteristic of these cell types in this species.

A large size range (12.4\(\mu m\) to 23.4\(\mu m\)) was associated with monocytes in this study. However, this is not uncommon for monocytes since ranges of 16\(\mu m\) to 25\(\mu m\) are routinely reported for these cells in eutherian species (Banks, 1981). The mean tammar wallaby monocyte diameter of 16.0\(\mu m\) falls within the ranges reported for a number of other marsupial species such as the possum (mean 15.0\(\mu m\), range 11.6-18.2\(\mu m\); Barbour, 1972), the koala (mean 17.0\(\mu m\), range 15.6-18.6\(\mu m\); Canfield and Dickens, 1982), the dunnarts, *S. crassicaudata* and *S. macroura* (mean 14.8\(\mu m\), range 12-20\(\mu m\); Haynes and Skidmore, 1991) and the Allied Rock-wallaby (mean 14.4\(\mu m\), range 11.3-18.7\(\mu m\); Spencer and Speare, 1992).

In eutherian mammals, the peroxidase enzyme is routinely found in the primary or azurophilic granules of monocytes and neutrophils (Root and Cohen, 1981). Peroxidase activity of a small number of cultured, but not freshly isolated, tammar wallaby monocytes was detected in the current study. This staining was unexpected, since in eutherian mammals, the maturation of monocytes into macrophages is usually accompanied by the loss of peroxidase. Perhaps the few cells isolated in these preparations were a small minority of peroxidase-positive monocytes that were not detected in routine assays. This requires further investigation since there is variability amongst marsupial species regarding the presence of this enzyme. Brushtail possum monocytes are peroxidase negative (Barbour, 1972) and in the South American opossum, myeloperoxidase positive blood cells are predominantly eosinophils, not monocytes (Armstrong and Ferguson, 1997). It is possible that the ability to generate toxic radicals via the peroxidase system may be different between marsupial and eutherian species and this may account for some of the variability in immune responses reported between these groups.
In this study, levels of monocytes present in the tammar wallaby differential counts may have been underestimated due to the similarity in morphological forms between plasma cells and monocytes. Monocytes are known to exist in a binucleated form (Zucker-Franklin et al., 1981) and since morphology was the main criterion for the classification of cell types in the current work, binucleated cells may have been incorrectly attributed to the lymphocyte group. However, the possibility of this is small, since studies undertaken on the isolation and culture of monocytes (see Chapter 4) resulted in low levels of these cells being isolated from peripheral blood which agrees with the differential counts recorded in Table 3.4.

Absolute monocyte counts for the tammar wallaby were between $0.06 \times 10^6$ cells/mL and $0.20 \times 10^6$ cells/mL which is similar to the red-necked wallaby ($0 \text{ to } 0.23 \times 10^6$ cells/mL; Hawkey et al., 1982) but is less than that of the Allied Rock-wallaby ($0 \text{ to } 0.84 \times 10^6$ cells/mL; Spencer and Speare, 1992).

**Neutrophils**

At the light microscope level, tammar wallaby neutrophils possessed similar staining characteristics to those described for other mammals (Schalm et al., 1975) and were of a similar size to cells of the brush-tail possum (Barbour, 1972) and Allied Rock-wallaby (Spencer and Speare, 1992). However, the mean diameter for these cells of 12.4μm was less than the minimum diameter of 12.6μm reported for koalas (Canfield and Dickens, 1982) which is most likely an anomaly with the koala study where large lymphocytes were also a common finding. Fine granules that most likely represented the azurophilic, primary granule population were clearly visible in the cytoplasm of some tammar wallaby cells.

A number of different nuclear arrangements were recorded in tammar wallaby neutrophils, from immature band cells through to highly lobulated mature neutrophils. Tammar blood rarely contained greater than 5% neutrophils as immature cell forms, which is consistent with studies of domestic eutherian species (Banks, 1981). In contrast, up to 11% of neutrophils comprised immature cells in dunnart species (Haynes and Skidmore, 1991). These cells included those with annular nuclei, band cells and cells with less than three nuclear lobes (Haynes and Skidmore, 1991). The
unidentified cells such as those shown in Figure 3.1h were presumed to be of granulocytic origin because of their staining characteristics and phagocytic ability. These cells were therefore most likely granulocytic blast cells since immature ‘left shift’ neutrophils, those with reduced nuclear lobulation, most often appeared with these cells.

The majority of tammar wallaby neutrophils possessed between four and seven nuclear lobes. In humans, greater than five nuclear lobes is termed ‘neutrophil hypersegmentation’ and is associated with immune disorders such as anaemias and pyogenic infections (Lewis, 1977). However, increased nuclear lobulation has previously been reported as routine in the blood of various marsupial species such as the brushtail possum (Barbour, 1972), the red-necked wallaby (Protemnodon rufogrisea; Hawkey et al., 1982), two species of dunnart, S. macroura and S. crassicaudata (Haynes and Skidmore, 1991) and in both the blood and milk of the koala (Canfield and Dickens, 1982; Young and Deane, 2001). Hypersegmentation is also an artefact of aging blood (Schalm et al., 1975). Together, these findings suggest that in marsupials, multilobed structures are not uncommon and should not be interpreted as being pathologically significant in the absence of other supporting evidence.

Absolute neutrophil counts for the tammar wallaby were between $1.01 \times 10^6$ cells/mL and $2.41 \times 10^6$ cells/mL, which is well within the range for clinically normal macropods (Hawkey et al., 1982). A large range of these cells have been reported for marsupial mammals and mean neutrophil levels of $0.6 \times 10^6$ to $3.4 \times 10^6$ cells/mL for the brushtail possum (Buddle et al., 1994), between $0.55 \times 10^6$ cells/mL to $12.21 \times 10^6$ cells/mL for the Allied Rock-wallaby (Spencer and Speare, 1992), $0.66 \times 10^6$ to $3.90 \times 10^6$ cells/mL for the red-necked wallaby (Hawkey et al., 1982) and $3.5 \times 10^6$ cells/mL for the koala (Canfield and Dickens, 1982) are considered normal for these animals.

At the ultrastructural level, tammar wallaby neutrophils are similar in morphology to koala (Canfield and Dickens, 1982), dunnart (Haynes and Skidmore, 1991) and eutherian cells (Borregaard and Cowland, 1997). In humans, primary neutrophil granules are an electron dense, homogeneous population that measure approximately 0.4μm in diameter. Secondary granules generally measure less than 0.3μm, are more
abundant, and may contain a crystalloid inclusion (Zucker-Franklin et al., 1981). In
the present study, most tammar wallaby neutrophil granules measured between
0.13μm and 0.44μm which is consistent with the size of granules in human cells. An
unusually shaped granule, the dumbbell-shaped granule, was common in tammar
wallaby neutrophils (Figure 3.7). These structures have also been recorded in human
neutrophils (Zucker-Franklin et al., 1981) and the eosinophils of some eutherian
species such as camels and sheep (Johnson et al., 1999). Dumbbell-shaped granules
are no longer considered true granules as they are thought to be segments of smooth
endoplasmic reticulum with an as yet unknown function (Zucker-Franklin et al.,

Other organelles present in tammar neutrophils included obvious Golgi apparati and
stretches of RER. However, in some cells, there were few or no visible mitochondria,
ribosomes or RER. These differences may be attributed to sectioning artefacts or
variability in the age of the cells, with the more mature forms being less
biosynthetically active.

In the present study, freshly isolated tammar wallaby neutrophils stained positively
for the peroxidase enzyme. The presence of peroxidase in these cells is consistent
with the findings in eutherian neutrophils, where myeloperoxidase (MPO) is the major
heme protein. In eutherian neutrophils, MPO reacts with hydrogen peroxide and
halide ions to produce bactericidal oxidative molecules (Cooray et al., 1993). The
findings of the present study agree with reports from eutherian mammals, but are not
consistent with previous marsupial studies that reported difficulties with detecting
myeloperoxidase in the cells of M. domestica but found that peroxidase staining was
consistent and reliable in another granulocytic cell type, the eosinophil (Armstrong
and Ferguson, 1997).

In the present study of tammar wallaby neutrophils, variability in peroxidase
positivity was evident but this was addressed by analysing granulocytes within a total
leukocyte population in order to avoid excess processing steps involved in the
isolation of neutrophil populations that may have contributed to the breakdown of the
enzyme. Another factor that may have contributed to inconsistent peroxidase
detection was their possible pre-activation prior to analysis. Pre-activated cells may
release their granule contents so that they are not detected in routine staining procedures.

**Eosinophils**

Tammar wallaby eosinophils were clearly distinguished from other cell types by their prominent orange-pink staining granules. When treated with Giemsa stain, the nuclei of these cells stained a distinct blue colour and were easily differentiated amongst the granules. In common with the eosinophils of eutherian mammals, cells from the tammar possessed both unilobed and bilobed nuclei that were not covered by the large granules. Diameters of these cells were typically large (10.5μm to 16.8μm) and within the reported range for both eutherian (Schalm et al., 1975; Banks, 1981) and metatherian species such as the brushtail possum (Barbour, 1972), the koala (Canfield and Dickens, 1982) and the Allied Rock-wallaby (Spencer and Speare, 1992).

The presence of ribosomes, small profiles of RER and mitochondria within tammar wallaby eosinophils suggests that these cells are biosynthetically and metabolically active. The size and shape of granules was consistent with those found in koalas (Canfield and Dickens, 1982), humans (Zucker-Franklin et al., 1981) and sheep (Yamada and Sonoda, 1970) but smaller than those of camels (Johnson et al., 1999). There was a variation in granule size but all granules appeared to be similar in composition. There was no evidence of a separate microgranule population such as has been reported in humans and camels (Parmely and Spicer, 1974; Johnson et al., 1999). Granules from tammar wallaby and koala eosinophils have an electron dense matrix and lighter staining crystalloid core which is different from dunnarts that have ovoid, elongated smaller granules mixed with larger, spherical granules with electron dense cores (Haynes and Skidmore, 1991). These differences may be due to different processing conditions, but may also reflect variations in chemical composition of the granule cores.

In general, when eosinophils are detected in the peripheral blood of marsupials, they occur in higher numbers than those reported for most eutherian mammals (Banks, 1981). Svensson et al. (1998), in a haematological study of the chuditch (*Dasyurus geoffroii*), reported eosinophils levels of 6.1% and in the Allied Rock-wallaby, where eosinophils were identified in 40% of blood smears, 4.3% of these were at levels
greater than 5% of the differential count (Spencer and Speare, 1992). These levels most likely reflect parasitic burdens in free-living animals, since high levels of eosinophils (up to $10^7$ cells/mL) are commonly associated with parasitic infections in humans (Hoffman et al., 2000). The tammar wallabies sampled in the present study were subjected to annual worming treatments and this may have reduced parasite loads. Levels of eosinophils detected in the peripheral blood of these animals were routinely less than 3.5% of the differential count.

The comparatively low eosinophil differential count in this study is mirrored in the calculated absolute eosinophil counts. Tammar wallaby peripheral blood contained between $0.06 \times 10^6$ to $0.16 \times 10^6$ eosinophils/mL. This is considerably lower than other marsupials such as the brushtail possum ($0.09 \times 10^6$ to $0.53 \times 10^6$ cells/mL; Buddle et al., 1994), the Allied Rock-wallaby ($0 \times 0.68 \times 10^6$ cells/mL; Spencer and Speare, 1992) and the red-necked wallaby ($0 \times 0.66 \times 10^6$ cells/mL; Hawkey et al., 1982).

**Basophils**

Basophils were identified at low levels ($\leq 1\%$ of the differential count) at the light microscope level and with the exception of one cell that was difficult to classify (Figure 3.9), were not detected in electron microscopy preparations. Tammar wallaby basophils are similar in appearance to those described for other marsupial species. Like the tammar, the red-necked pademelon (*Protemnodon rufogrisea*; Hawkey et al., 1982), the koala (Canfield and Dickens, 1982) and the eastern quoll (*D. viverrinus*; Canfield, 1998), all have cells that contain small, dark blue to purple-staining granules that cover the nucleus and are also found scattered throughout the cytoplasm. The mean diameter of these cells in the current study was 14.0µm, which is consistent with brushtail possum and koala basophils that measure 14.2µm (Barbour, 1972; Canfield and Dickens, 1982).

Marsupial haematology studies routinely report the lack of basophil cell types in peripheral blood analyses (Parsons et al., 1971; Haynes and Skidmore, 1991; Svensson et al., 1998) although levels of up to 6% of the differential count were recorded for some Allied Rock-wallabies (Spencer and Speare, 1992). The low levels of basophils recorded in the present work are consistent with other mammalian studies
where basophils are not often documented (Schalm et al., 1975; Banks, 1981). Given the low incidence of these cell types, it is possible that reports suggesting that some species of marsupials do not possess basophils are perhaps attributable to small sample sizes rather than to a true absence of this granulocyte. Lighter-staining basophils may also be easily confused with dark-staining neutrophils after treatment with aged differential stains. This may also account for the apparent absence of these cells in some reports.

Absolute basophil counts for the tammar wallaby were between 0 to 0.07 x 10^6 cells/mL which is in agreement with results obtained for the red-necked wallaby (0 to 0.03 x 10^6 cells/mL; Hawkey et al., 1982) but different from basophil counts for the Allied Rock-wallaby that had a maximum level of 0.75 x 10^6 cells/mL (Spencer and Speare, 1992).

**Erythrocytes**
The current study was not concerned with investigation of haematology values associated with tammar wallaby erythrocytes. However, the morphology of red blood cells was routinely observed during differential leukocyte counts. The size, staining characteristics and morphology of these cells was consistent with other marsupial studies (Ponder et al., 1929; Haynes and Skidmore, 1991), with levels of Howell Jolly bodies and nucleated RBCs similar to those reported for other marsupials (Hawkey, 1975) and those of feline and equine blood (Banks, 1981). In mammals, these parameters generally indicate the increased production of erythrocytes which is consistent with results obtained for one animal in this study that contained 50% nucleated RBCs in the differential count and was later found at autopsy to have internal injuries.

**Total Leukocyte Counts**
Commentary on the significance of total and relative cell numbers for marsupial species must be considered in the light of what is currently known about normal ranges for these animals. In the present study, numbers of peripheral blood leukocytes from *M. eugenii* were examined over a three year period to establish meaningful reference values for this species. Absolute leukocyte counts for individual cell populations were calculated for nine animals tested in this study and in
In this study, there was no obvious correlation between total leukocyte counts of tammar wallabies and incidences of births, sex, reproductive status or season. However, there was a trend for younger animals and those animals born at the Macquarie University Fauna Park, to have the highest leukocyte counts (>5 x 10^6 cells/mL). Since these values were within the normal published ranges for marsupial species, this relationship may not be significant. However, it is noteworthy that many of the study animals were relocated from Cowan (NSW) three months prior to this work and the translocation stresses associated with such a move may have influenced leukocyte levels (Baker et al., 1998).

In marsupials, total leukocyte counts of greater than 10 x 10^6 cells/mL have been associated with infection (Hawkey et al., 1982), stress (Baker et al., 1998) or excitement (Canfield and Dickens, 1982). The average values and ranges of total leukocyte counts for the Marsupialia fall outside the normal range compared with humans (Hawkey, 1977), with a mean cell count that is generally lower than that of domestic species such as dogs, sheep and guinea pigs (Banks, 1981). White blood cell numbers in the peripheral blood of healthy eutherians are generally between 5 x 10^6 and 10 x 10^6 cells/mL. An abnormally low level of white blood cells, often
associated with viral infection, is termed leukopenia and occurs in humans when peripheral blood leukocyte numbers fall below 4 x 10^6 cells/mL (Lewis, 1977). Four of the tammar wallabies in the present study possessed mean leukocyte levels less than 4 x 10^6 cells/mL and a number of others had raw counts that fell at or below this level at times during the study. Whilst it is important to set critical values such as these on a species-specific basis, it is interesting to note here the possible effects of viral loads on haematological parameters for later discussion. Differences in in vitro immune responses within the study colony suggest that low leukocyte counts (<5 x 10^6 cells/mL) may indicate depressed immunity. These findings are discussed in Chapter Five.

**Leukocyte Ratios**

The ratio of lymphocytes to neutrophils has been used in a number of reports by marsupial researchers as an indicator of stress or disease. Baker and Gemmel (2000) reported captive possums to have a high lymphocyte to neutrophil ratio in health and found a correlation between decreased L:N ratios and possum fatalities. Lymphocyte numbers ranged from 6.77 x 10^6 to 12.01 x 10^6 cells/mL and neutrophil counts ranged from 0.95 x 10^6 to 3.15 x 10^6 cells/mL in this study. Animals that subsequently died showed varied changes in leukocyte numbers, from 17.9 x 10^6 cells/mL (comprising 10.5 x 10^6 lymphocytes/mL and 7.5 x 10^6 neutrophils/mL in one male animal, to 8.6 ± 1.4 x 10^6 total leukocytes in female animals, comprising lymphocyte numbers of 5.3 ± 1.4 x 10^6 cells/mL and neutrophil counts of 3.1 ± 0.7 x 10^6 cells/mL). In both sets of fatalities, the L:N ratio was decreased when compared with healthy animals.

Neutrophilia was successfully demonstrated to correlate with disease in marsupials in a study of mammals suffering from bacterial infections (Hawkey and Hart, 1987). Conversely, Wells et al. (2000) noted that healthy animals from brushtail possum colonies also contained comparatively high levels of neutrophils.

The comparison of relative L:N or N:L numbers appears to be most relevant when 'normal' values have been established for the particular species in question. In tammar wallabies, lymphocytes were the most predominant cell type and most animals possessed a N:L greater than 1.5. This disagrees with the findings of Spencer and Speare (1992) for the Allied Rock-wallaby, where they suggest that a N:L ratio of
less than 1.0 is routine for marsupial species. Others have suggested that the N:L ratio is reversed in immature animals (Hawkey, 1975). In the present study, L:N% were considered low when less than 60:40 (ratio ≤1.5) and this was the case with animals 12F5094T, 1008, 1400, 1652, 1844 and 209. Results from functional analyses of cells from these animals appeared to confirm that the capacity to generate an *in vitro* immune response may have been compromised and this is discussed in Chapters 4 and 5.

Differences in the interpretation and applicability of relative leukocyte counts have been dealt with in a number of different ways. Obendorf (1983) and Canfield *et al.* (1989) chose to establish absolute numbers rather than ratios since they recognised the large variability that is often present in differential cell counts, which is not always consistent with the degree of difference between absolute cell numbers. Hawkey *et al.* (1982) suggest that morphology rather than actual number of neutrophils is a more appropriate diagnostic tool for detecting changes in immune status. Hypolobulation, loss of granules and development of basophilic cytoplasm were indicative of bacterial infection in the red-necked wallaby even when cell counts were normal. In tammar wallabies, 'left shift' cell types were evident in some samples but hypolobulation and changes in staining characteristics were not evident. The lack of granules between and within samples in the present study was attributed to day-to-day variations in staining since there was no obvious relationship between cell numbers and the presence or absence of neutrophil cytoplasmic granules.
<table>
<thead>
<tr>
<th>Species</th>
<th>Total Leukocyte Count (x10⁶ cells/mL)</th>
<th>Differential Cell Count</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby (<em>Macropus eugeni</em>)</td>
<td>4.5</td>
<td>74</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>40.0</td>
<td>50.9</td>
</tr>
<tr>
<td>Long-nosed potoroo (<em>Potorous tridactylus</em>)</td>
<td>8.1</td>
<td>68.5</td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>3.4-11.0</td>
<td>31</td>
<td>51</td>
</tr>
<tr>
<td>Allied rock-wallaby (<em>Petrogale assimilis</em>)</td>
<td>9.0</td>
<td>56</td>
<td>41.2</td>
</tr>
<tr>
<td>Quokka (<em>Setonix brachyurus</em>)</td>
<td>11.0</td>
<td>52</td>
<td>43</td>
</tr>
<tr>
<td>Red Kangaroo (<em>Macropus rufus</em>)</td>
<td>7.0</td>
<td>27</td>
<td>69</td>
</tr>
<tr>
<td>Agile Wallaby (<em>Macropus agilis</em>)</td>
<td>4.9</td>
<td>33.5</td>
<td>56.7</td>
</tr>
<tr>
<td>Goodfellow’s tree kangaroo (<em>Dendrolagus goodfellowi</em>)</td>
<td>6.8</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>Brushtail possum (<em>Trichosurus vulpecula</em>)</td>
<td>13.4</td>
<td>50.2</td>
<td>43.3</td>
</tr>
<tr>
<td>Koala (<em>Phascolarctos cinereus</em>)</td>
<td>8.1</td>
<td>52.0</td>
<td>42.5</td>
</tr>
<tr>
<td>Opossum (<em>Didelphis virginia</em>)</td>
<td>7.2</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Chuditch (<em>Dasyurus geoffroii</em>)</td>
<td>8.4</td>
<td>18</td>
<td>71</td>
</tr>
</tbody>
</table>

* only present in one animal
NR none reported

Table 3.7: Total and Differential Leukocyte Counts of Selected Marsupial Species. (l) lymphocytes, (n) neutrophils, (m) monocytes, (e) eosinophils, (b) basophils. Numbers are presented as the mean of reported ranges and rounded to one decimal place for the purposes of comparison.
Immunohistochemistry

The phenotyping of individual cell types within lymphocyte test populations has relied principally on results of functional responses to plant mitogens to infer T and/or B cell identities (Ashman et al., 1976; Wilkinson et al., 1992b; Baker et al., 1998). These functional assays are described elsewhere in this study and were used principally to demonstrate the in vitro functional capacity of the test cell populations. In the present study, histochemical staining and morphological inspection using both light and electron microscopy were used to establish preliminary identification of leukocyte populations of the peripheral blood. However, further analyses were necessary in order to identify different subpopulations of cells, particularly lymphocytes, that were very similar at the light microscope level. Thus, species cross-reactive antibodies such as anti-CD3 and anti-CD5, that recognise T cells within tammar wallaby lymphoid tissue (Hemsley et al., 1995), were used to develop a method that would facilitate identification and quantitation of tammar wallaby peripheral blood T lymphocytes.

There is only one published study of blood cell immunolabelling in marsupials, that of the koala, where a polyclonal anti-human CD3 antibody was used to identify T cells using the technique of fluorescence activated cell sorting (Wilkinson et al., 1995). In that study, between 54 and 73% of peripheral mononuclear cells demonstrated reactivity with this species cross-reactive antibody. In the present study, similar results were also obtained for tammar wallaby lymphocytes when a polyclonal anti-CD3 antibody was used (Table 3.6). However, results obtained for immunolabelling with the anti-CD5 antibody were lower than this, between 25 and 49% of the mononuclear cell population. Whilst these reduced values may be due to lower T cell numbers, this difference in staining properties between the two antibodies may be explained by a difference in expression pattern between the two cell surface antigens. The CD5 antigen is expected to be present at lower levels since unlike the CD3 antigen, it is not expressed in the cytoplasm before cell surface expression (Rose et al., 1992). The tammar wallaby cells labelled with anti-CD3 antibody in the current work were stained diffusely throughout the cytoplasm, whereas anti-CD5 positive cells were stained most strongly in the cell membrane. Provided that the age and storage conditions of the freshly prepared lymphocyte slides were controlled, results
from this study demonstrate that antibodies used successfully on tammar wallaby lymphoid tissue can also be used to discriminate T cells in the peripheral blood.

CD3 and CD5 are both considered pan T cell markers in eutherians (Roitt et al., 1998). CD3 is associated with the T cell receptor and CD5 is implicated in the regulation of second messenger molecules and is thought to play a role in the modulation of the cellular immune response. Thus, whilst both of these cell antigens are characteristic of T cells, their expression may be dependent on different cell functions. In this study, the staining properties of the CD5 antigen using the mCD5 antibody were more distinct when compared with those of the polyclonal anti-CD3 antibody. Variations in intensity and distribution of staining within the cytoplasm of blood lymphocytes may represent different levels of expression of the CD5 antigen which is consistent with reports of regulation of expression of this marker in mice (Azzam et al., 1998). This may, in turn, reflect the presence of subpopulations of lymphocytes at various stages of development and activation. There was no direct correlation between differential cell counts and the levels of lymphocyte staining. However, higher levels of T lymphocytes were identified in samples with low lymphocyte levels which suggests that a compensatory mechanism may be in operation to ensure optimum levels of total lymphocytes are present irrespective of proportions in differential counts.

Previous immunocytochemical studies of mammalian peripheral blood cells, including those of the koala (Wilkinson et al. 1995), have been successful in identifying the CD3 T cell antigen on blood lymphocytes but have not reported the detection of the pan T cell marker, CD5. As well as the T cell surface, this antigen is also co-expressed on the surface of B cells in some species (Kipps, 1989). However, with the exception of rabbit B lymphocytes that all co-express this antigen, CD5 is present on less than five percent of total lymphocytes in humans and cattle (Appleyard and Wilkie; 1998). Previous immunohistological studies of tammar wallaby and Eastern grey kangaroo lymphoid tissue have confirmed that the mCD5, mCD3 and pCD3 antibodies all stain lymphoid tissue in areas that are consistent with the distribution of T cells (Hemsley et al., 1995; Old and Deane, 2001). The evidence to date suggests that the anti-CD5 antibody does not label a large proportion of B cells in marsupials and certainly too few to significantly affect the values obtained in the
current study. Thus, the monoclonal anti-CD5 antibody (CD5/54) can be reliably used as a T cell marker for peripheral blood lymphocytes of marsupials.

**Incidental Blood Findings**

A number of blood-borne organisms were detected during the course of this study and whilst further investigation of these entities was outside the scope of the present work, morphological inspection revealed similarities in structures to the cysts of *T. gondii*, coccidian oocysts and an unidentified flagellate. The presence of unsporulated *T. gondii* cysts in the circulatory system of mammals usually only occurs in cases of disseminated disease (Yamaguchi, 1981) so it is noteworthy that at the time of writing, animal 1400 was still living and apparently in good health, suggesting a successful recovery from toxoplasmosis. Alternatively, it is possible that this organism was present as a result of sample contamination since the bleeding site, near the base of the tail, is often in contact with the soil, which in turn, is contaminated with animal faeces. Whether carried in the blood or introduced at the time of sampling, the presence of the cyst indicates contamination of the study group environment with this organism. Toxoplasmosis and coccidiosis is known to have affected animals from the Macquarie University Fauna Park (personal communication Professor Desmond Cooper of Macquarie University and Dr. Catherine Bernard of University of Western Sydney), therefore cysts resembling those generated by protists were not unexpected findings in the blood of a small number of these animals. However, whilst the presence of blood parasites has been previously reported in marsupials (Mackerras, 1959) and positive seroconversion to *T. gondii* antigen by animals in the Macquarie University colony has been investigated (personal communication Prof. D. W. Cooper), there have been no accounts of blood-borne flagellated organisms detected in these animals. The presence of the unidentified flagellate in animal 12F5094T is noteworthy because this animal was subsequently found dead from an undetermined cause. The presence of these blood-borne organisms and their affect on the immunocompetency of tammar wallabies clearly requires further investigation.

**3.4 Conclusion**

Tammar wallaby peripheral blood leukocytes were similar in size, appearance and numbers to those of other vertebrate mammals. Specifically, total leukocyte counts
for this species were on the lower end of normal compared with eutherian mammals and fell within the range reported for other marsupials. At both the light and electron microscope level, tammar leukocytes were similar in basic morphology to eutherian mammals and other marsupials studied to date with the exception that the peroxidase enzyme was not detected in monocytes and lysozyme granules appeared to be absent from these cells. T lymphocytes were distinguished from other lymphocyte populations using an immunocytochemical slide technique that is suitable for use on small or rare blood samples such as those from rare and endangered species, for example the Rufous hare-wallaby and the Long-footed potoroo.
CHAPTER FOUR

Isolation, Characterisation and Functional Capacity of Tammar Wallaby Phagocytic Cells

4.1 Introduction

Mammalian phagocytic cell populations include both mononuclear agranular cells and polymorphonuclear granulocytes (Morel et al., 1991). Upon exposure to antigen, these innate effector cells become activated, migrate to the infected site and proceed to isolate and neutralise foreign pathogens or damaged tissue in the process of phagocytosis. This process requires an increase in oxygen consumption and ATP generation known as the respiratory burst response (Morel et al., 1991). Once inside the monocyte or granulocyte, antigens are usually destroyed by oxidative species generated within the phagocytic vacuole (Beaman and Beaman, 1984). Despite these cell defences, intracellular pathogens such as the Mycobacteriaceae can be harboured inside the monocytic phagocyte and survive for long periods of time (Bermudez and Petrofsky, 1997). Moreover, there is some evidence to suggest that the more robust response of granulocytes may be successful in neutralising these bacteria if they are mounted at the time of initial infection (Ogata et al., 1992).

To date, with the exception of the detection of myeloperoxidase (MPO) in possum and opossum granulocytes, there are no reports of the bactericidal properties of phagocytic cells from marsupial mammals or the in vitro responses of marsupial granulocytes. Reports of the isolation and culture of macrophages are limited to the culture of alveolar (Wedlock et al., 1996) and peritoneal (Moriarty and Thomas, 1986) macrophages from the brushtail possum undertaken as part of larger experiments evaluating mycobacterial responses.

The production of bactericidal chemicals and the phagocytic behaviour of myeloid cell lines can be studied in vitro provided that the desired cell populations can be isolated and cultured. Neutrophils are relatively easy to isolate from most mammalian blood samples and since these cells are quick to respond to the presence of antigens, their responses are readily studied in vitro. Monocytes and macrophages are more
difficult to isolate since they form a small part of a larger mononuclear cell population. These cells are slower to respond to activation stimuli than granulocytes and also require more stringent culture conditions. They are thus less easily studied in vitro.

In order to allow characterisation of the innate responses of marsupial phagocytes, it was first necessary to establish a method for the isolation and culture of both monocyte and granulocyte cell populations. This section describes these isolation techniques as well as the assessment of the in vitro phagocytic and antimicrobial responses of these cells.

4.2 Results

4.2.1 Isolation and Culture of Phagocytic Cells

4.2.1.1 Polymorphonuclear Cells: Neutrophils, Eosinophils and Basophils

4.2.1.1.1 Isolation

A number of different methods were assessed for their ability to isolate granulocytic cell populations. Criteria used for the assessment of successful isolation included cell viability, cell yield, the ease with which RBCs were removed from cell preparations and the amount of pre-activation caused by the isolation protocol. Early studies used the large polymer Dextran-500 to separate granulocytes from RBCs by sedimentation (see 2.3.4.2.4). Granulocytes settled as a buffy layer above RBCs and were recovered after hypotonic lysis of erythrocytes. This method was successful in purifying granulocyte populations, but it entailed lengthy preparation times and reduced cell recoveries. In some cases, isolated granulocytes possessed morphology associated with pre-activation and in others, decreased cell viabilities were obtained when compared with other protocols.

The double layer ficoll-paque 1.077/1.119 density gradient separation technique (Method 2.3.4.2.4c) was successful in the isolation of granulocyte populations. However, low levels of RBCs and monocytes were present when using this protocol. This labour intensive procedure was thus abandoned for use during this study.

High cell yields and viabilities were obtained by recovering the sedimented cells from beneath the ficoll 1.077 layer and above the RBC layer formed during isolation of
PBMC from whole blood (Method 2.3.4.2.1). This procedure was used for the majority of assays performed in this study. Hypotonic lysis (2.3.4.1) was used for the removal of RBCs in granulocyte preparations since the use of the ammonium RBC lysis solution was not efficient in removing the high numbers of residual erythrocytes contained in these preparations. After RBC removal, cells were washed with PBS, HBSS, HBSS- or culture media before final resuspension in HBSS-. Cells washed in media provided the largest and most consistent responses, so granulocytes were routinely washed with RPMI 1640 media with or without serum (depending on assay requirements) prior to a final wash and resuspension in HBSS-. HBSS- proved to be the most effective suspension medium since the absence of calcium and magnesium ions prevented neutrophil aggregation in vitro (Wright, 1988). This solution was adopted for routine use unless otherwise indicated. Granulocytes prepared using the 1.077 ficoll-histopaque isolation method were routinely more than 90% viable (2.3.3.3.2) and contained ≤10% eosinophils when inspected after Diff-Quik staining (see 2.3.3.1).

4.2.1.1.2 Polarisation
Polarisation responses occur as physical changes in the morphology of cells after they are exposed to uniform gradients of potential chemotactic factors (Haston and Shields, 1985). After treatment with polarising agents, contractile fibres within cells rearrange in response to a locomotion stimulus (polarisation), but cells do not undertake directional movement. Once polarisation has occurred in response to antigens in vivo, leukocytes become activated to undergo chemotaxis and cross blood cell walls in order to migrate into tissue environments where they perform their major functions (Bray, 1992). Before assessment of the chemotactic abilities of tammar wallaby granulocytes was undertaken, test agents known to promote polarisation responses in eutherian animal cells were investigated for their stimulatory effects on these cells. This was undertaken by inspection of the change in cellular morphology of granulocytes after they were co-cultured with treatment agents (Method 2.3.7.4.1).

Freshly isolated untreated granulocytes were routinely spherical in shape and were devoid of cytoplasmic extensions (Figure 4.1a). The cell membranes of these cells appeared smooth and did not contain visible granules. Resting, unstimulated cells were generally smaller than activated granulocytes. In contrast, the size and shape of
granulocytes changed in response to test agents. In suspension, these morphological changes included a ruffled, non-spherical membrane surface and the adoption of bipolarity that often involved the appearance of a leading front or uropod (Figure 4.1b and c). Cells appeared flattened and granules were clearly visible in the cell cytoplasm (also Figure 4.1b). Treatment agents that induced these changes included LPS at 10µg/mL and 100µg/mL, 1/10 dilutions of bacterial culture supernatants (S. aureus and E. coli), a 1/10 dilution of fresh serum and serum-opsonised zymosan (SOZ) at 0.5mg/mL.

In these assays, LPS at 100µg/mL caused polarisation of a larger proportion of cells than did 10µg/mL treatments with this bacterial agent (see Figure 4.2). However, bacterial culture supernatants caused higher polarisation responses than either of the levels of LPS (Table 4.1). The formyl peptide, formyl-methionyl-leucyl-phenylalanine (fMLP) did not induce polarisation of tammar wallaby granulocytes when tested at concentrations of 10⁻⁷M.
Figure 4.1: Differential Interference Phase Contrast Micrographs of Tammar Wallaby Granulocytes. Cells were fixed in 2.5% gluteraldehyde after treatment with polarising agents. Figure (a) clearly illustrates the spherical morphology of control cells after 30mins incubation at 37°C in humidified air containing 5% CO₂. Figure (b) shows the morphology of a cell treated with a 10% dilution of supernatant obtained from a culture of *Staphylococcus aureus*. The polarised cell has a constriction (arrow) and a leading lamellopodium (l). Figure (c) is representative of many polarised cells after treatment with LPS. These cells have ruffled cytoplasm and have lost their spherical morphology.
Figure 4.2: Polarisation Responses of Tammar Wallaby Granulocytes. Duplicate samples of cells were incubated with and without test agents for 30mins at 37°C in humidified air containing 5% CO₂ before fixation with 2.5% gluteraldehyde. After treatments, at least 200 cells were scored for polarisation morphology using phase contrast microscopy. Data presented here is the mean result from two animals. Bars represent ± range/2.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Response</th>
<th>Control</th>
<th>Serum (1/10)</th>
<th>SOZ (1/10)</th>
<th>fMLP 10^{-7}M</th>
<th>S. aureus supernatant (1/10)</th>
<th>E. coli supernatant (1/10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400</td>
<td>% Unpolarised</td>
<td>96.5</td>
<td>11.9</td>
<td>3.9</td>
<td>94.5</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>% Polarised</td>
<td>3.5</td>
<td>88.1</td>
<td>96.1</td>
<td>5.5</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>12F3CODT</td>
<td>% Unpolarised</td>
<td>94.4</td>
<td>21.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>% Polarised</td>
<td>5.6</td>
<td>79.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1844*</td>
<td>% Unpolarised</td>
<td>68.6</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>15.8</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>% Polarised</td>
<td>31.4</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>84.2</td>
<td>90.4</td>
</tr>
<tr>
<td>1E722CFT*</td>
<td>% Unpolarised</td>
<td>74.2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>11.7</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>% Polarised</td>
<td>25.8</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>88.3</td>
<td>88.7</td>
</tr>
</tbody>
</table>

**Table 4.1: Polarisation Responses of Tammar Wallaby Granulocytes.** Mean results for four separate animals are presented here from duplicate counts of at least 200 cells in suspension using phase contrast microscopy. Cells were incubated with and without polarisation test agents for 30 mins at 37°C in humidified air containing 5% CO₂ before fixation with 2.5% gluteraldehyde. * cells held at 4°C prior to treatments. SOZ, serum opsonised zymase; fMLP formyl peptide; NT – Not tested due to insufficient cell numbers.
4.2.1.3 Chemotaxis

Granulocytes are directed along chemotactic gradients when migrating to the site of injury or infection (Downey, 1994). In order to assess the in vitro ability of marsupial granulocytes to migrate and respond to known chemotactic agents, the directional movement of tammar wallaby granulocytes was assessed using modified versions of both the under-agarose technique and the Boyden chamber assay (2.3.8.4.2).

A preliminary study in which experimental protocols were established for the under-agarose assay was undertaken as part of a supervised undergraduate research project by S. Flecknoe (UWS, Sydney). In this work, granulocytes were isolated by ficoll-paque density gradient centrifugation (Method 2.3.4.2.1), RBCs were removed by hypotonic lysis and cells were placed in buffered salt solution and held at 4°C until required (holding time < 2 hours). During this time, agarose beds (0.3 - 0.7% agarose in HBSS containing phenol red) were poured onto cleaned microscope slides and allowed to set. A series of three wells of between 0.2 and 0.4mm diameter were cut into the set agarose to allow placement of the test cells between a control and a treatment well. Before placement into wells, cells and treatment agents (LPS- and nutrient-broth-recovered supernatants from cultures of both E. coli and S. aureus) were brought to room temperature (or 37°C). Results from this work demonstrated that granulocytes responded to the S. aureus using this under-agarose method. In the present study, these initial results were confirmed and extended to include other test agents and practical modifications to the experimental protocol. Using the under-agarose technique, neutrophil polarisation induced by a chemotactic gradient was very apparent even in microscopic fields inspected adjacent to the well containing cells alone, where little directional cell motility had occurred. However, quantitation of these responses was cumbersome, so the technique was modified. Changes to the protocol included a change of substratum from a microscope slide to a 6-well tissue culture plate. This change was undertaken so that agarose was poured into, rather than onto, the support structure to control drying of the agarose bed, a factor that caused inconsistent results in earlier studies. Culture time was also extended from two hours to overnight to ensure that treatment agents had effectively diffused through the agarose so that cells were given sufficient exposure time to test agents.
Consistent with preliminary studies, granulocytes moved towards and into the well containing the *S. aureus* test solution. Little migration occurred towards the control well containing a solution of sterile nutrient broth, the growth media for the *S. aureus* preparation. Despite these modifications, the under-agarose procedure was still labour intensive and results were difficult to quantify, therefore a modified Boyden chamber assay technique was also performed (Method 2.3.7.4.2). Minimal migration through 3μm membrane filters was observed in the absence of chemoattractant factors using this technique. When chemoattractant factors were added to the bottom well, the number of cells that migrated through the filter towards these factors visibly increased. Cell numbers were difficult to count at the base of the tissue culture vessel after migration, so detection of the numbers of cells that migrated through the filter into the base wells was assessed using the MTT assay (2.3.7.1.1). This assay detected the presence of viable cells by colourimetric measurement of the amount of solubilised reduced formazan salt present after mitochondrial processing of substrate MTT. Comparison of OD values between control and stimulated test wells was therefore used as an indicator of relative cell numbers (Table 4.2).

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Optical Density of Migrated Cells (OD*±R/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1657</td>
</tr>
<tr>
<td>fMLP (10^-6M)</td>
<td>0.057±0.003</td>
</tr>
<tr>
<td></td>
<td>1EF4CB2T</td>
</tr>
<tr>
<td></td>
<td>0.046±0.003</td>
</tr>
<tr>
<td></td>
<td>0.044±0.003</td>
</tr>
<tr>
<td></td>
<td>13788BAT</td>
</tr>
<tr>
<td></td>
<td>0.067±0.018</td>
</tr>
<tr>
<td></td>
<td>0.051±0.011</td>
</tr>
<tr>
<td></td>
<td>0.107±0.068</td>
</tr>
</tbody>
</table>

**Table 4.2: Migration of Tammar Wallaby Granulocytes.** The MTT assay was applied to granulocytes after their migration through 3μm filters for indirect quantitation of cell numbers. Results are reported as duplicates ± range(R)/2. *Optical Density at 550nm.

*S. aureus* and *E. coli* supernatants were strong chemoattractant factors in this assay. LPS also demonstrated some chemoattractant properties. Similar to the findings in the polarisation assay, fMLP at 10^-7M did not cause measurable chemotaxis of cells isolated from animals 8021 and 1994 (data not shown). However, 10^-6M fMLP caused significant (*P*<0.05) migration of cells from animal 13788BAT and a non-significant migration for cells from animals 1657 and 1EF4CB2T (Table 4.2).

A 1/10 dilution of stock zymosan activated serum (ZAS) was also chemoattractive for granulocytes from animals 8021, 12F3CODT and 1994. A 1/50 dilution caused the
migration of more cells than the 1/10 dilution. LPS at 100μg/mL was chemotactic but was less so than ZAS. Both heat-inactivated and fresh serum were identified as a chemoattractant for tammar wallaby granulocytes, although there was some variability in responses of cells from different animals. Cells isolated from animal 8021 showed a decreased response to serum when compared with cells from animal 1994.

In summary, tammar wallaby granulocytes were successfully isolated from anticoagulated whole blood using ficoll-paque density gradient separation medium. These cells showed significant polarisation and chemotactic responses to test agents prepared using gram positive and gram negative bacteria. However, the formyl peptide, fMLP, caused inconsistent responses in these assays. Test agents containing serum also stimulated strong polarisation responses when used in isolation or after activation by the yeast-derived product, zymason.

4.2.1.2 Monocytes and Monocyte-Derived Adherent Cells

4.2.1.2.1 Isolation

A number of different techniques were used to isolate monocytes and monocyte-derived adherent cells from tammar wallaby peripheral blood.

Whole Blood

To achieve the maximum experimental material from blood samples, early experiments were performed using a 1:1 and a 1:5 dilution of anticoagulated whole blood in RPMI-1640 and QBSF®-51 serum-free media. These solutions were treated with antibiotics (2.3.1.3) that included the antifungal agent amphotericin B, to limit the growth of contaminants that would ordinarily be removed by routine wash steps in other separation methods. The diluted blood solutions were placed directly into a tissue culture flask. Of these assays, whole blood cultures in QBSF®-51 media supported the growth of macrophages. However, this initial result was not reproducible and also supported the growth of contaminating epithelial/endothelial cells that could not be separated from the macrophage population. Therefore, this method was considered unsuitable for the establishment of routine monocyte cultures.
Density Gradient Media
Tammar wallaby monocytes and monocyte-derived adherent cells were isolated with other mononuclear cells using density gradient centrifugation (see 2.3.4.2.1). A number of different density gradient media were investigated for their ability to isolate the maximum numbers of monocytes from whole blood samples. A discontinuous gradient formed by layering Percoll medium of different densities (Method 2.3.4.2.1b) was assessed for its ability to enrich mononuclear cell fractions for monocytes. This result was successful, but large blood volumes (>15mL) were required for recovery of isolated cells in sufficient yield for analysis. The recovery of cells was also affected by the need to increase wash steps to remove residual percoll retained within the small monocyte enriched cell fraction.

Monocytes were also isolated as part of the PBMC preparations recovered from whole blood using density gradient separation media of densities 1.077g/mL and 1.083g/mL. Both of these separation media were successful in the isolation of monocytes after adherence of the PBMC to tissue culture plates (Method 2.3.4.2.2). However, increased numbers of adherent cells were recovered using the higher density medium, Histopaque 1.083. As expected, the total cell numbers recovered were dependent on the numbers of monocytes in the original samples, which varied between 0.6 x 10^5 to 2.0 x 10^5 cells/mL of whole blood (see Table 3.5). Contamination with granulocytic cells was one drawback of this technique. This problem was overcome by the continued culture of cell preparations, since granulocytes are short-lived cells that do not survive for long periods in culture. Both Ficoll and Histopaque 1.077 and Histopaque 1.083 density gradient media were used for the isolation of monocytes in this study.

4.2.1.2.2 Culture
A further aim of this work was to isolate and develop procedures for maintaining cultures of monocyte-derived cells for future studies. To this end, a number of factors were investigated for their ability to support the growth of these cell lines.

Culture Media
Serum-supplemented and serum-free media were assessed for their ability to promote and sustain monocyte culture and support cell maturation in vitro. RPMI 1640 alone
or supplemented with 5% and 10% FBS and QBSF®-51 serum-free media were trialled in this study. Increased numbers of adherent cells were present in cultures grown in QBSF®-51 serum-free media. Cells grown in this system displayed spreading morphology, resulting in adherence to the culture vessel. In contrast, cells incubated in standard culture media used successfully for eutherian monocyte culture such as serum-supplemented RPMI-1640, showed little adherence and very little spreading morphology.

When cultured in QBSF®-51 serum-free medium, adherent cells remained viable for up to 20 days. However, it was necessary to co-culture these cells with lymphocytes (as PBMC) or with a 1:1 ratio of serum-free media to lymphocyte conditioned serum free media (LCSFM; 2.3.4.2.2) after the monocyte adherence phase in order to maintain continued viability of the adherent cell population (see Figure 4.3). After 24 hours, for the PBMC preparation, suspension cells were removed and media was changed to include only QBSF®-51 and antibiotics. For the adherent cells in media supplemented with LCSFM, media was replaced with a fresh 1:1 serum-free media: LCSFM solution. After a further three days in culture, 50% of the culture volume was removed and replaced with fresh media. This process was repeated every 5 days and supported the continued growth and development of monocytes and monocyte-derived cells.

Other Factors

Experiments included a variety of culture vessels, such as petri dishes, small and large diameter tissue culture wells (96, 48, 24, 12 and 6 wells) and two sizes of tissue culture flasks (50 and 150mL). A number of different culture times for monocyte adherence were also attempted. Results from these experiments suggested that 50mL tissue culture flasks were optimal for monocyte culture. In these experiments, 30 – 60 min incubations were too short to enable all cells to adhere to the substratum. Increasing the adherence time to 90-150mins proved optimal for mononuclear cell adherence. Longer incubation times resulted in adherence of large numbers of contaminating lymphocytes. Therefore, routine monocyte isolation experiments were performed in 50mL tissue culture flasks using 120mins adherence times.
Cell harvesting

The numbers of adherent cells that remained in 96-well culture vessels after monocyte adherence was between $1 \times 10^4$ and $1.4 \times 10^5$ cells/well as assessed indirectly by the MTT assay (2.3.7.1.1). These cell numbers were too low for assessment of monocyte function and therefore the recovery of improved yields of test cells was a necessary requirement of this work. The harvesting of cells from tissue culture vessels was thus a critical step in the ability to recover sufficient cell numbers for further analyses. A number of methods were assessed for their efficiency in the recovery of adherent cells. Careful scraping with a sterile cell-scraper followed by short, sharp tapping of the culture vessel proved to be the most successful harvest method. The viability of cells recovered with this technique was $>70\%$. Alternative techniques also trialled included a commercially prepared Cell Dissociation Solution (Sigma) that was unsuccessful in removing cells from the culture vessel. Recovery of cells using standard EDTA/trypsin methods (Doyle and Griffiths, 1998) resulted in low cell yields and cell viabilities of less than 40\%.

These cells were not easily removed from the culture vessel for assessment of viability using the trypan blue technique, so viability was established within the culture vessel using the MTT assay (Method 2.3.4.2.2). The presence of a blue MTT formazan precipitate within monocytes indicated the presence of active mitochondria within these cells. When treated with MTT in situ, the majority of monocytes and monocyte derived cells cultured using the conditions described above were viable (see figure 4.5b). This technique was used since it could be performed in situ and did not require the recovery of cells as is necessary for trypan blue exclusion testing.

4.2.1.1.1 Cellular Morphology

When monocytes were successfully cultured as described above, cells within these cultures varied in shape and size. In general, cells increased in size and measured between 10-15\(\mu\)m and 80\(\mu\)m (at their longest projection) after the first 3-5 days in culture. After the initial culture period, cells with morphology similar to eutherian macrophages were apparent. These cells appeared as large, granulated, spherical structures that were visibly flattened against the culture flask substratum (Figure 4.4c). Other forms of these cells adopted a ‘fried-egg’ appearance with a flattened anterior portion and a long posterior cell process (Figure 4.4a). Cells that developed
spindle-shaped or stellar morphology, with large, dendritic-like processes extending from the cytoplasm were also apparent (Figures 4.4b and d). Other monocytic cells forms, with perinuclear pale staining regions similar to plasma cells, were also identified in these experiments (see figure 4.5a). Multinucleated cells (Figure 4.6) were identified in cultures of monocyte-derived cells when these were co-cultured with lymphocytes or when the culture was supplemented with LCSFM.

![Image](image.png)

**Figure 4.3: Inverted Phase Micrograph of Adherent Cells Co-cultured with Lymphocytes.** These cells were cultured as PBMC for 5 days in serum free media. Note the dead lymphocytes and the large macrophage-like cells (arrows). One of these cells appears to be engulfing a dead lymphocyte (arrow-head at bottom left of picture).

4.2.1.2.3 Mitogen Stimulation of Monocytes
A small number of experiments were undertaken to assess the response of peripheral blood monocytes to stimulation with the plant lectin Con A and the bacterial agent, LPS.

i) Treatment with Con A
Monocytes (approximately 0.6 x 10^6 cells/well) were treated with Con A at 25μg/mL in QBSF®-51 serum-free medium and cultured for 48 hours at 37°C in humidified air containing 5% CO₂ (standard conditions). Con A-treated cells maintained their viability when compared with control cells after this time and while a proliferation trend was apparent at higher concentrations of this mitogen, this proliferation was not statistically significant. In all, Con A did not appear to be cytotoxic in this system.
Figures (b) and (d) are most likely monocyte-derived dendritic cells. Figure (b) has the characteristic stellar shape associated with dendritic cells and both cells possess the finger-like projections and size that are used to identify these cell types. Figure (c) is a monocyte-derived macrophage that has retained its spherical morphology after adherence.

Figure 4.4: Phase contrast images of tammar adherent cells in culture. Cells of various morphologies were identified in cultures of adherent cells from PBMC preparations. Figure (a) is a Diff-Quik stained monocyte-derived adherent cell. Note the typical macrophage ‘fried-egg’ appearance of this cell. The nucleus is stained pink.
Figure 4.5: Tammar Wallaby Mononuclear Cells. Figure (a). Cells resembling plasmacytoid monocytes were identified in Diff-Quik stained adherent cell preparations cultured in QBSF-51® serum-free media. Note the clear staining perinuclear region (arrow). Figure (b) illustrates viable adherent mononuclear cells in culture as indicated by the deposit of a blue MTT formazan precipitate. Note the characteristic horseshoe shape of the monocyte (arrow).
**Figure 4.6: Tammar Wallaby Multinucleated Giant Cells.** Multinucleated giant cells appeared in PBMC cultures after 3-4 days in serum-free media. Figure (a) is a phase contrast image of a large adherent cell that has ingested a dead or dying cell from within the culture environment. Figure (b) is a Giemsa-stained brightfield image of a typical binucleated cell. Figure (c) is a Diff Quik stained cell with three visible nuclei (arrows). Cell debris is visible in the background.
ii) Treatment with LPS

Monocytes were treated with 10μg/mL LPS in QBSF®-51 serum-free media and cultured overnight under standard conditions. Cells within these cultures displayed signs of deterioration such as ziosis or ‘blebbing’ of the cell membrane (Bignold, 1992) and vacuolisation of the cell cytoplasm (see Figure 4.7). Therefore, LPS was judged to be cytotoxic for monocytes under these conditions.

![Image of cell morphology](image)

**Figure 4.7. Effect of Lipopolysaccharide Treatment on the Morphology of Tammar Wallaby Adherent Cells.** Addition of LPS at 10μg/mL in QBSF®-51 serum-free media caused membrane blebbing (small, spherical or elongated surface projections produced under the influence of toxins - arrow) and vacuolisation of the cell cytoplasm after 24hrs in culture.

In summary, recovery of cells using the histopaque 1.083 density gradient medium resulted in the highest yields of monocytes and mononuclear adherent cells from small volumes (<10mL) of tammar wallaby peripheral blood. When these cells were cultured in QBSF®-51 serum-free medium supplemented with lymphocyte conditioned media or co-cultured with lymphocytes, cells resembling macrophages, plasmacytoid monocytes and dendritic cells developed within 3-5 days. Macrophage populations remained viable for up to 20 days in this culture system.

### 4.2.2 Phagocytosis

The behaviour of eutherian phagocytes in vitro is often used as an indication of the functional immune capacity of these cell populations (Jefferies et al., 1996). In the present study, tammar wallaby monocytes and granulocytes were investigated for
their ability to phagocytose a variety of inert and antigenic particles to confirm their in vitro phagocytic properties. Latex beads, non-viable yeast cells and viable bacterial cells were all assessed for their ability to promote in vitro phagocytic responses.

**Monocytes**

Monocytes and monocyte-derived adherent cells demonstrated positive phagocytic responses when incubated with 1.1µm latex beads and when co-cultured with lymphocytes. Figure 4.8a is an example of a monocyte that had ingested a large number of 1.1µm latex beads. Numbers of ingested particles were difficult to count due to their small size and aggregation within the cell body. However, monocytes routinely ingested >10 beads per cell when cultured in media containing serum. No studies of latex bead ingestion were performed in serum-free media.

Figure 4.9 shows cultured monocyte-adherent cells ingesting dead or dying lymphocytes. Adherent cells, especially those of dendritic morphology, appeared to become stimulated when co-cultured with bacteria or dead or dying lymphocytes. Cells with dendritic cell morphology were routinely observed to engulf dead and dying cells when incubated with lymphocytes (also Figure 4.9). Macrophages and dendritic cells matured to large actively phagocytosing cells within 36 hours of culture.

**Granulocytes**

Tammar wallaby granulocytes ingested all particles used for assessment of phagocytic capacity in this study, although there was a difference in the numbers of particles ingested. These differences appeared to be related to the size and origin of the particles and whether serum was present in the assay system. Figures 4.8b and 4.8c are photomicrographs of the ingestion of bacterial cells. Figure 4.8b is a neutrophil that ingested *S. xylosus*, a bacterium previously isolated from a tammar wallaby pouch (Old and Deane, 1998). This cell was identified in a smear prepared from the lysis of a 1:1 culture of whole blood and QBSF®-51 serum-free media. Figure 4.8c is a photomicrograph of a neutrophil that had ingested *S. aureus*, also after co-incubation of whole blood with the bacteria.
Granulocytes also ingested 1.1μm and 3μm latex beads. Figure 4.8d shows a group of ingested 3μm latex beads within a phagocyte from a whole blood culture. In this phagocytic study where beads were added directly to whole blood and incubated under standard conditions for 4.5 hours, 68% of adherent cells contained ingested beads, with the range of beads ingested being 0-7 beads/cell (mean = 2.4 beads/cell). However, adherent cell numbers were low (n=25), which is consistent with the small numbers of leukocytes that would be contained within a smear of whole blood. Despite these limitations, this assay demonstrated the ability of cells within whole blood to spontaneously ingest foreign particles without the need for particle opsonisation.

Figure 4.8e is an H and E stained preparation of granulocytes showing the ingestion of 3μm serum-opsonised latex beads within an eosinophil and a neutrophil. Serum-opsonised 3μm latex beads were ingested by granulocytes at numbers up to 8 beads per cell when incubated for periods up to 5.5 hours. To confirm that beads were ingested rather than simply adherent to the cell cytoplasm, slides were washed with xylene to remove extraneous beads (Lehnert and Tech, 1985). Although this harsh treatment washed some cells off the slide so it could not be used for further quantitative studies, this method did verify the intracellular nature of the bead ingestion. Within the granulocyte population, both neutrophils and eosinophils ingested 1.1 and 3μm beads. These cells were differentiated by staining with H and E in order to confirm the phagocytic behaviour of marsupial eosinophils. A further benefit derived from H and E staining was the removal of extraneous beads from the cell surface without lifting the cells from the slides.

In culture systems not containing serum either in the pre-wash media or as the opsonin, the number of particulates ingested was considerably lower, with no phagocytosis occurring in many cells. Similarly, when granulocytes from two animals were cultured in HBSS- without media or serum supplementation, 3μm latex beads were not ingested. In contrast, when cells from the same animals were cultured in RPMI, ingestion of 3μm beads was evident at up to 7 beads/cell (mean = 3 beads/cell; n=30). The mean numbers of cells ingesting latex beads were further increased when beads were opsonised with serum (mean = 5 beads/cell; n=42).
In mixed cultures of monocytes and granulocytes, monocytes ingested more of the smaller latex beads in fewer cells than did granulocytes, which ingested less than 10 beads per cell. In these assays, the numbers of ingested beads appears to be related to the volume that the beads occupied within the cell. Hence, larger numbers of smaller beads were ingested for the monocytes, since these cells were generally larger than neutrophils.

In summary, tammar wallaby monocytes and granulocytes displayed phagocytic behaviour as assessed by the uptake of latex beads, yeast, bacteria or dead and dying lymphocytes when exposed to these particles in vitro. In these experiments, larger numbers of particles were ingested in the presence of serum than without, suggesting an important role for serum opsonisation in tammar wallaby phagocytic responses.

4.2.3 Antimicrobial Responses

4.2.3.1 Oxidative Responses

The oxidative burst responses of monocytes and granulocytes were assessed in response to a number of different treatment agents using a number of different methods. The different assay techniques were used to differentiate the nature of the oxidative response in order to more clearly define the capacity of these cells to respond to exogenous stimuli.

Monocytes and Monocyte-Derived Cells

Preliminary experiments with adherent cells recovered from PBMC preparations demonstrated an increase in cellular activity in the presence of 50, 100 and 500μg/mL of LPS as assessed by the MTT assay (2.3.7.1). Although cell numbers were limited (see earlier results), a small study of oxidative burst responses in these cells was carried out in an attempt to establish optimum conditions for future studies.
Figure 4.8: Phagocytosis of Latex Beads and Bacteria by Peripheral Blood Cells of the Tammar Wallaby. Figure (a) is a monocyte that has ingested many serum-opsonised 1.1\(\mu\)m latex beads. Figure (b) is a neutrophil that has ingested *Staphylococcus xylosus* bacteria previously isolated from a wallaby pouch. Figure (c) shows the ingestion of *Staphylococcus aureus* by a neutrophil. Figure (d) shows the phagocytosis of 3\(\mu\)m latex beads by an unidentified phagocyte isolated from whole blood. Figure (e) is an H and E stained smear of granulocytes that have ingested 3\(\mu\)m opsonised latex beads. Scale Bar = 5 \(\mu\)m.
Figure 4.9: Inverted Phase Micrographs of Adherent Cells Co-Cultured with Lymphocytes. Figures (i) – (iii) show a number of adherent cells that have phagocytosed dead lymphocytes in a 5 day culture of PBMCs in serum free media. Note the large macrophage-like cells and the ingested dead lymphocytes (arrows).
**NBT Assay**

Tammar wallaby PBMC-adherent cells were cultured from animal IE722CFT (2.1 x 10^5 cells/well) and animal 1994 (1.1 x 10^5 cells/well) in 48 well rather than 96 well plates in an effort to increase the number of monocytes recovered from PBMC preparations. Cells were treated with PMA at 1μg/mL for 30mins to trigger the respiratory burst. Visual inspection of cultures after this time confirmed the presence of a light blue precipitate in test wells indicating a positive response, but this response was not detectable when the optical density of the dissolved blue precipitate was measured compared with control cells (Method 2.3.7.4.4). A further experiment with monocytes from animal 1844 (7.2 x 10^4 monocytes/well) was trialled, where cells were treated with both LPS at 50μg/mL and PMA at 1μg/mL. Again, optical densities of control and treated cells were not significantly different. However, visual inspection of wells demonstrated a positive PMA response visible as deposits of blue formazan (see Figure 4.10).

**Cytochrome C Assay**

The Cytochrome C assay (2.3.7.4.5) in conjunction with superoxide dismutase was performed on monocytes isolated from animal 1994. After incubation with PMA at 1μg/mL for 30mins, no cytochrome C response was detected. However, a further 20 min incubation detected an increase in oxidative response with the OD values of PMA treated cells increasing to 0.045 ± 0.007 compared with control cells at 0.034 ± 0.003. This increase in OD was not apparent in wells containing superoxide dismutase (SOD), which suggests that superoxide was produced by monocytes in response to PMA. The necessity for increased incubation times may be a reflection of the slower response kinetics of these cells or the limits of detection of the assay when using these relatively low cell numbers.

**Granulocytes**

**4.2.3.1.1 NBT Assay**

Two different forms of NBT assay were carried out on tammar wallaby granulocytes (see Methods 2.3.7.4.4). In the first of these tests, cells were applied to microscope slides as either whole blood or isolated granulocyte populations and scored manually for NBT responses. The second assay involved the culture of granulocytes in
microtitre plates and spectrophotometric quantitation of the amount of NBT produced by granulocytes in response to oxidative burst stimulants.

**NBT Slide Assays**

The NBT slide test was useful for qualitative and semi-quantitative detection of the oxidative burst response of cells in whole blood samples as members of a total leukocyte population, or in purified granulocyte cell preparations. In these assays, samples of blood or isolated cells were applied directly to microscope slides and incubated under standard conditions for 30 min to facilitate adherence of phagocytic cell populations to the slide. Slides were then treated with yellow NBT solution with and without the stimulating agents LPS at 100μg/mL or 1μg/mL PMA, followed by counterstaining with safranin to allow detection of the blue NBT formazan precipitate formed within cells as a result of a positive oxidative burst response. Variable responses were obtained with PMA. However, stimulation with LPS caused an increase in oxidative burst response in all samples tested. Responses of strongly positive cells included a 1.5 to 2-fold increase in cell size and deposition of the NBT formazan precipitate within the entire cytoplasmic area of the cell (see Figure 4.11b). In control assays, where NBT was applied to cells without treatment agents, small areas of blue staining were visible in many of the cells (see Figure 4.11a). These responses are most likely due to a slight increase in respiratory activity stimulated by adherence to the slide substrate (Johnson et al., 1992).

As well as routine analyses on freshly isolated blood, this test was also performed on tammar wallaby whole blood samples aged 26 hours in order to simulate the conditions that would be encountered when transported blood samples were analysed. This work was necessary since the NBT slide assay was to be used on samples that would arrive from interstate and would be subject to this length of delay before reaching the laboratory (see Chapter 7). Results from these assays verified the effectiveness of this test for aged blood samples, with intense blue NBT deposits easily identified in monocytes and granulocytes within the whole blood samples after treatment with LPS at 50μg/mL.
**Figure 4.10: Oxidative Burst Response of Tammar Wallaby Mononuclear Cells.** Figure (a) shows NitroBlueTetrazolium (NBT) staining of adherent cells treated with NBT only and figure (b) shows the same cells treated with the oxidative burst stimulus, Phorbol Myristate Acetate, also in the presence of NBT. Note the deposit of blue formazan in figure (b) demonstrating the increased oxidative burst response.
The NBT test was also used in conjunction with some phagocytic assays to identify the cellular location of the respiratory burst response. In these assays, NBT was added to the culture medium at the same time as phagocytic particles. When inspected using inverted phase microscopy, NBT formazan deposits were intensified at the site of both *S. aureus* and latex bead ingestion.

*NBT Colourimetric Assay*

In order to compare the degree of oxidative burst responses caused by different treatment agents, quantitation of the amount of NBT reduced by granulocytes was undertaken following the method of Rook *et al.* (1985). Granulocytes were isolated using Method 2.3.4.2.4 and where sufficient cell numbers were available, these cells were incubated with a number of test agents known to stimulate the respiratory burst response of eutherian granulocytes (Badwey and Karnovsky, 1980). The majority of analyses were performed using a 60 min culture period, although some experiments were conducted using the shorter culture period during the optimisation phase of this assay. For all test agents, optimum respiratory burst responses were achieved when cells were washed with media and cultured in HBSS-. Washing and culturing with HBSS- resulted in low oxidative burst responses (Table 4.3).
Figure 4.11: NBT Staining of Tammar Wallaby Granulocytes. After adherence to microscope slides, granulocytes were incubated with yellow NBT solution or NBT solution containing LPS. A positive oxidative burst response was indicated by formation of a blue NBT-formazan precipitate. Control cells are visible in (a). Note the blue focal staining of polymorphonuclear cells, most likely due to activation by adherence to the slides. In figure (b), after stimulation with LPS at 100µg/mL, cells are enlarged and possess darkly stained NBT-formazan regions. All cells are counterstained with safranin.
Figure 4.12: Representative Nitroblue Tetrazolium (NBT) Standard Curve. Known quantities of NBT were added to wells and the Optical Density of the dissolved formazan precipitate was recorded at 655nm. The quantity of reduced NBT per $10^5$ cells was calculated from the equation $y = 0.7094x - 0.0368$ generated from the above standard curve.
Quantitation of NBT was conducted using Method 2.3.7.4.4 (NBT Colourimetric Assay). Cell numbers varied in these assays dependent on the amount of peripheral blood sample available for testing. Thus, to facilitate comparisons between treatments, all results were converted to the quantity of NBT reduced for every $10^5$ cells (after Cross et al., 1996) using the standard curve shown in Figure 4.12. Values for resting, unstimulated NBT-treated granulocytes varied between 3.2μg/$10^5$ cells and 283.5μg/$10^5$ cells (mean 77.1μg, SD 91μg). One human sample analysed in this study produced 209.7μg reduced NBT/$10^5$ cells which fell within this range. The very large standard deviation appeared to reflect differences within individual animal responses as well as responses between different animals and may also have been influenced by the different levels of pre-activation evident across the range of isolation procedures employed in this study. This variation was standardised by expressing the values for treated versus untreated cells as the Respiratory Burst Index (RBI) where the quantity of reduced NBT for treated cells was divided by the quantity of reduced NBT for control cells in the same experimental set. RBIs equal to 1.0 indicated no differences in NBT production from control cells, RBIs < 1.0 indicated an inhibition in the production of oxidative products and a RBI > 1.0 indicated an increase in the respiratory burst response to treatment agents.

The results for quantitative NBT analyses on granulocytes are summarised in Table 4.3. LPS at 10μg/mL and 100μg/mL consistently caused an increase in the respiratory burst response. Concentrations below and between these values also increased baseline NBT responses but these were not consistent and appeared to be animal-dependent. Other stimulatory agents also promoted the oxidative burst response, but these responses were less consistent between animals. PMA at 1μg/mL caused increased NBT reduction in 3 out of 5 animals tested, with one animal showing no significant change (1612) and one displaying a depressed response (1652). fMLP was tested at concentrations of $10^{-6}$M, $10^{-7}$M and $10^{-8}$M and only one animal (1960) showed an increase in oxidative response to these treatments. Higher concentrations ($10^{-6}$M) of this formyl-peptide produced a depressed response in cells from animals 1657 and 8021, which most likely indicates that fMLP is cytotoxic for tammar wallaby granulocytes when applied at these concentrations.
The yeast-derived particulate, zymosan, caused an oxidative burst response when used at 0.5μg/mL. Zymosan-activated serum was also stimulatory when used at 1/10 dilutions of stock preparations on cells from animals 1844 and 8021. The effects of serum were also apparent when used alone or in combination with particulate agents. Serum-opsonised bacteria caused increases in RBIs with opsonised *E. coli* having the most pronounced affects. However, this response appeared to be dose-dependent since lower dilutions of these agents did not result in significant oxidative responses (Table 4.3).

4.2.3.1.2 Superoxide Dismutase

The production of superoxide anions by tammar wallaby granulocytes was distinguished from other respiratory burst products by treatment of control cells and cells plus stimulating agents, with superoxide dismutase using the cytochrome C assay (Method 2.3.7.4.5). In this assay, addition of the enzyme superoxide dismutase suppressed the production of superoxide anions and thus allowed the identification of treatment agents that specifically promoted the production of this anion. Cells were treated with LPS (100μg/mL), PMA (1μg/mL) and fMLP (10⁻⁵M) with and without SOD. In all cases of granulocyte stimulation with PMA, detection of a respiratory response by granulocytes was abolished by the presence of SOD, showing specificity of this response for superoxide anions (Table 4.4). In contrast, LPS and fMLP did not produce the superoxide anion under the conditions of the test.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment agent</th>
<th>Respiratory Burst Index (RBI)</th>
<th>Treatment agent</th>
<th>Respiratory Blast Index (RBI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1657</td>
<td>M1L(10^-5M)</td>
<td>1.01</td>
<td>8021</td>
<td>M1L(10^-5M)</td>
</tr>
<tr>
<td>1652*#</td>
<td>M1L(10^-5M)</td>
<td>0.92</td>
<td>1657</td>
<td>M1L(10^-5M)</td>
</tr>
<tr>
<td>1652*#</td>
<td>M1L(10^-5M)</td>
<td>0.82</td>
<td>1844</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1652*#</td>
<td>M1L(10^-5M)</td>
<td>0.78</td>
<td>8021</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1906*</td>
<td>M1L(10^-5M)</td>
<td>0.97</td>
<td>1400</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1906*</td>
<td>M1L(10^-5M)</td>
<td>1.05</td>
<td>1400</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1906*</td>
<td>M1L(10^-5M)</td>
<td>1.03</td>
<td>1583</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1906*</td>
<td>M1L(10^-5M)</td>
<td>1.65</td>
<td>13788BAI</td>
<td>PMA(100μg/ml)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.63</td>
<td>1400</td>
<td>E-coli serum (1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.34</td>
<td>1400</td>
<td>E-coli serum (1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>0.90</td>
<td>1844</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.27</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>0.84</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.66</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.66</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.51</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.52</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
<tr>
<td>1612</td>
<td>LPS(10μg/ml)</td>
<td>1.35</td>
<td>8021</td>
<td>SOZ(1/10)</td>
</tr>
</tbody>
</table>

Table 4.3: Respiratory Blast Indices of NBT-Treated Granulocytes. See text for details. *5min incubation, #10min incubation.

#HSSS-media.
Animal and Cell Treatment
(Optical Density at 550 nm)

<table>
<thead>
<tr>
<th></th>
<th>1652</th>
<th>12F3CODT</th>
<th>1994</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+LPS</td>
<td>+PMA</td>
<td>+LPS</td>
</tr>
<tr>
<td>control</td>
<td>0.083±0.003</td>
<td>0.084±0.003</td>
<td>0.085±0.003</td>
</tr>
<tr>
<td>-SOD</td>
<td>0.082±0.006</td>
<td>0.132±0.002</td>
<td>0.084±0.004</td>
</tr>
<tr>
<td>+SOD</td>
<td>0.081±0.003</td>
<td>0.086±0.003</td>
<td>0.086±0.006</td>
</tr>
</tbody>
</table>

Table 4.4: Cytochrome C Assay Optical Density Results for Stimulated Tammar Wallaby Granulocytes. Cells were treated with stimulants (100μg/mL LPS; 1μg/mL PMA and 10^−6 M fMLP) and cytochrome C with and without superoxide dismutase (SOD). Control, untreated cells were incubated in parallel with treated cells to assess the effect of the treatment agents. After incubation for 10mins, optical densities (OD) of the supernatants were measured at 550nm (referenced to 655nm). Results are means of triplicate values. Results for treatments with PMA were significant at P<0.05 for cells from animals 1652 and 12F3CODT.

4.2.3.1.3 Reactive Nitrogen Species

The ability to produce reactive nitrogen species as part of the phagocytic oxidative defence system is an important part of eutherian innate defences (Pithon Curi et al., 1998). Therefore, these responses were also investigated in tammar wallaby phagocytic cell populations. A standard curve for nitrite concentration (Method 2.3.7.4.7) was constructed using sodium nitrite standards between 0 and 100μM. The limit of detection using this assay was 0.5μM when 100μL of culture supernatant was assayed. Results reported here are expressed as μM nitrite accumulated in culture supernatant as determined indirectly from the standard curve shown in Figure 4.13. Most control cells tested in this study produced measurable levels of NO. Since the OD of culture media was affected by incubation, a standard curve in media was prepared for each assay time and a representative curve is shown in Figure 4.13.

Monocytes

Nitrite levels in monocyte supernatants were assayed with and without LPS and PMA treatments. Monocyte nitrite responses were difficult to assess due to the apparent slower kinetics and the low cell numbers recovered during isolation steps. Monocytes were routinely recovered as adherent cells from PBMCs after a two-hour incubation in 48 well tissue culture plates and subsequent removal of suspension cells. Cells from tammar wallabies 8021, 1657 and 1E722CFT were incubated with and without 1μg/mL LPS and
control and treatment cell supernatants were assessed for the presence of secreted nitrite as described in Method 2.3.7.4.7.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cell Number (cells/well)</th>
<th>Control (µM nitrite)</th>
<th>LPS (1µg/mL) (µM nitrite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8021</td>
<td>1.6 x 10^5</td>
<td>1.035 ± 0.070</td>
<td>1.350 ± 0.035</td>
</tr>
<tr>
<td>1657</td>
<td>3.6 x 10^4</td>
<td>1.590 ± 0.065</td>
<td>1.830 ± 0.178</td>
</tr>
<tr>
<td>1E7722CFT</td>
<td>2.7 x 10^4</td>
<td>1.490 ± 0.035</td>
<td>1.420 ± 0.035</td>
</tr>
</tbody>
</table>

Table 4.5: Concentration of Nitrite Accumulated in Tammar Wallaby Adherent-Cell Culture Supernatants. Results reported as mean of duplicates ± Range(R)/2. (R <10%).

As evident in Table 4.5, the change in concentration of NO in response to LPS was 315nM for animal 8021, 240nM for animal 1657, and -70nM for animal 1E7722CFT. Statistical analysis of this data was not performed due to small sample sets (n=2). However, the responses from cells isolated from animals 8021 and 1657 indicate a marked increase in nitrite production between control and treated cells.

PMA was also used to trigger the production of NO in monocytes. Adherent cell preparations from animal 1994 (2.3x10^4 monocytes) were incubated with and without PMA at 1µg/mL and the supernatant was collected and analysed for nitrite accumulation. Control cell production of nitrite was 340nM (from graph). PMA stimulated cultures produced 2979nM. It is interesting to note that this same culture system produced no oxidative burst response to PMA under the conditions of the test (10mins incubation) when tested using the superoxide dismutase assay (see Table 4.4).
Figure 4.13: Representative Standard Curve for Nitrite Determination in Culture Supernatants. Units of NO₂⁻ were estimated from a standard curve prepared from dilutions of 100μM sodium nitrite from between 0 and 100μM in increments of 10μM. One hundred microlitres of stock solutions were mixed with an equal volume of modified Griess reagent. The mixture was incubated at RT for 10mins after which the absorbance of each well was measured at 540nm. Standard curves for nitrite dissolved in both HBSS and QBSF®-51 serum-free media were constructed since both of these media were analysed for nitrite accumulation. This graph was constructed from data where nitrite was dissolved in serum-free media.
Granulocytes

Fewer granulocyte samples were assessed for their ability to produce nitrite. However, the capacity to generate nitrite in response to PMA (1μg/mL) stimulation was demonstrated in cells isolated from one animal (1994). When tested using Method 2.3.7.4.7, control cells (6.1x10⁵ cells/well) produced 12.771μM nitrite, which increased to 19.438μM when cells were stimulated with PMA.

4.2.3.2 Non-Oxidative Responses

4.2.3.2.1 Antimicrobial Proteins

Isolation of Granules and Functional Analysis

Granules and whole cell fractions were isolated from tammar neutrophils and eosinophils and were processed for acid-extractable protein and dialysed according to Method 2.3.7.4.8. Before processing, granules exhibited the characteristic green colour associated with heme proteins (Cooray et al., 1993), which suggested the presence of myeloperoxidase (MPO). After processing and crude size separation, granulocytic extracts were analysed for total protein as outlined in 2.3.8.4.8 (see Table 4.6).

<table>
<thead>
<tr>
<th>Fraction Number and Description</th>
<th>Total Protein μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5) 100-50K granule extract</td>
<td>21.8</td>
</tr>
<tr>
<td>(1) 50K-3K granule extract</td>
<td>20.3</td>
</tr>
<tr>
<td>(3) &lt;3K granule extract</td>
<td>4.9</td>
</tr>
<tr>
<td>(6) 100-50K cell extract</td>
<td>116.5</td>
</tr>
<tr>
<td>(2) 50K-3K cell extract</td>
<td>32.9</td>
</tr>
<tr>
<td>(4) &lt;3K cell extract</td>
<td>ND (&lt;1)</td>
</tr>
</tbody>
</table>

Table 4.6: Total Protein Content of Granule Extracts and Whole Cell Fractions from Tammar Wallaby Pooled Granulocytes.

ND not detectable.
Polyacrylamide Gel Electrophoresis (PAGE)

The range of proteins within the granule fractions and their approximate molecular weights were identified by PAGE after gels were stained with Silver Stain Plus (Bio-Rad Laboratories; California, USA) according to manufacturer’s instructions. After size separation, granule extract 5 stained positive for proteins at <50kDa and > 100kDa and granule extract 1 stained positive for proteins at <50kDa. Both whole cell extracts, samples 2 and 6, stained strongly for the presence of protein of all sizes. Fractions 3 and 4 did not possess visible protein bands, which is consistent with the low protein levels detected in these samples (Table 4.6).

<table>
<thead>
<tr>
<th>Material</th>
<th>E. coli (cfu)</th>
<th>% of control</th>
<th>S. aureus (cfu)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A*</td>
<td>532 ± 34</td>
<td>100 ± 6</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>405 ± 7</td>
<td>76 ± 2</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>464 ± 20</td>
<td>87 ± 4</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Control B*</td>
<td>525 ± 51</td>
<td>100 ± 10</td>
<td>580 ± 17</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>420 ± 35</td>
<td>80 ± 8</td>
<td>376 ± 45</td>
<td>65 ± 12</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>815 ± 42</td>
<td>155 ± 5</td>
<td>448 ± 17</td>
<td>77 ± 4</td>
</tr>
</tbody>
</table>

Mean colony forming units (cfu) are reported as ± R/2. *Experiments conducted on two separate occasions.

Table 4.7: Bactericidal Activity of Granule and Whole Cell Fractions. Between 5-10µg/mL of total protein from each granule or cell fraction was added to 1/10^6 dilutions of test bacteria grown to logarithmic phase. Bactericidal activity of these fractions was quantified by counting the numbers of viable colonies surviving after bacterial cultures were incubated at 37°C for 40mins with and without cell and granule extracts.

Granule Bactericidal Activity

Assessment of the bactericidal activity of granule extracts was only undertaken in duplicate due to the limited quantities of available test material. To enable direct comparison of bioactivity, a standardised quantity of total protein from each extract was added to test bacterial suspensions. These suspensions had been diluted 1/10^5 in PBS after bacteria were grown to logarithmic phase since bacterial cells were more
susceptible to the cytotoxic affects of the extracts in this growth phase (data not shown).

Of these cell extracts, Fraction 3 (granule extracts <3K) contained bactericidal components against *S. aureus* but not *E. coli*. In fact, growth of *E. coli* was enhanced with this extract. In contrast, Fraction 1 (50K-3K granule extract) contained components that were inhibitory to both gram-negative and gram-positive organisms but, different from Fraction 3, had a larger effect on the gram negative bacteria. The fractions containing larger molecular weight species, i.e. Fractions 5 (100-50K granule extract) and 6 (100-50K whole cell extract), were both bactericidal against *E. coli* but of these, the concentrated granule extract was the most inhibitory. In summary, gram-negative bacteria were affected by granule extracts >3K but not <3K. Gram-positive bacteria were affected by granule extracts up to 50K. Granule extracts >50K were not tested against *S. aureus* in this study.
4.3 Discussion
In this study, monocytes and granulocytes were successfully isolated from the peripheral blood of the tammar wallaby using density gradient separation techniques routinely employed for the isolation of eutherian phagocytic cells (Rose et al., 1992). Culture conditions were established for the assessment of non-specific immune function and, using these conditions, monocytes and granulocytes demonstrated the capacity to ingest inert and biological particles and to generate oxidative responses when stimulated with a range of respiratory burst triggers. Culture conditions of peripheral blood monocytes were further optimised to promote the differentiation of monocytes into macrophages and dendritic cells using a commercially prepared serum-free medium (QBSF®-51). Other cellular defence strategies identified in this study included investigation of the non-oxidative bactericidal capacities of neutrophils and eosinophils. Granules within tammar wallaby granulocytes contained molecules that were cytotoxic for both gram positive and gram negative bacteria. In general, tammar wallaby phagocytic cells possessed similar non-specific defences to those of eutherian cells.

Cell Isolation and Culture
The differences in density between the less dense, agranular mononuclear cells and the more dense, granular polymorphonuclear cells of tammar wallaby peripheral blood were exploited in this study in order to separate these two phagocytic cell populations from the same blood samples.

Granulocytes
A number of different protocols and density gradient media were assessed for their ability to recover the maximum numbers of viable granulocytes from peripheral blood samples. The double density isolation technique used to isolate PBMC and polymorphonuclear cells (PMC) (Method 2.3.4.2.4c) successfully isolated the target cell populations from tammar wallaby blood. This protocol has previously been used to recover leukocyte populations from the peripheral blood of dogs (Strasser et al., 1998). However, whilst this technique was useful in recovering the required cell populations from tammar blood, it was labour-intensive and time-consuming and was inconsistent in the numbers of residual RBCs that remained in cell preparations.

191
The optimum method for the isolation of granulocytes was to recover these cells from beneath a ficoll layer formed during density gradient centrifugation of whole blood (Method 2.3.4.2.1). An advantage of this system was that since this method was also routinely used for the isolation of PBMC, the maximum numbers of cells were recovered from whole blood in the fewest possible steps. This was important as blood volumes were limited and cell recovery was thus a priority.

**Monocytes**

Monocytes and macrophages form an essential part of the innate immune system with functions ranging from clearance of dead cells from tissue beds through to direct attack on bacterial and viral pathogens (Savill *et al*., 1989; Rosenwasser, 1992). Therefore, the development of a culture system that supported the growth and manipulation of marsupial monocytes *in vitro* was an essential part of the investigation of the functional immune capacity of these cells.

Monocytes represent only a small proportion of blood cells in tammar wallaby peripheral blood (up to 4% of total leukocytes: see Chapter 3) and are thus difficult to isolate from relatively small blood volumes. In this study, Percoll discontinuous density gradients were successful in the enrichment of PBMC cell fractions for monocytes but required large volumes of blood to recover sufficient cells for analysis. Other density gradient media used to isolate tammar wallaby blood monocytes were Ficoll 1.077 and Histopaque 1.077 and 1.083 (H-1.083), also used to isolate PBMC in other parts of this study (see Chapter 5). Of these, H-1.083 resulted in the recovery of the highest numbers of mononuclear adherent cells from peripheral blood. The requirement for this higher density medium suggests that tammar wallaby adherent cells are of a higher density than eutherian monocytes, which are routinely isolated using separation media with a density of 1.077g/mL (Hunt, 1987).

**Culture Media**

The culture of eutherian monocytes is traditionally undertaken in serum-supplemented media, usually RPMI-1640, after plastic adherence of the isolated PBMC population (Cross *et al*., 1996; Stabel *et al*., 1997; Barbuddie *et al*., 1998). Using this technique, adherent cell populations are assumed to be predominantly monocytes, although in some studies up to 30% of these cells are lymphocytes (Stabel *et al*., 1997). In the
present study, the culture of tammar wallaby monocytes in serum-supplemented media (RPMI+10% FBS) was less successful in supporting tammar wallaby adherent cell culture than a commercially prepared serum-free media (QBSF®-51) containing insulin, transferrin and BSA. Furthermore, to sustain viability and promote differentiation of monocytes into macrophages and dendritic cells, co-culture with lymphocytes within the first 24 hours was required. The ongoing viability and continued cell maturation of these cells also required addition of LCSFM to the culture medium.

A variety of distinct monocytic cell lines may be derived from mononuclear adherent cells cultured from peripheral blood. Amongst these are the more traditional monocyte-macrophage populations and the more recently described dendritic cell forms that are characterised by their morphologies since there are relatively few antibodies that differentiate these cell populations. Macrophages with morphology similar to that of eutherian cells were apparent in the culture system after 3-5 days incubation in serum-free media. Also present in these cultures were multinucleated giant cells that also make up approximately 5% of the total cells cultured from mononuclear cell populations of pig blood (Talbot et al., 1998). In eutherian mammals, giant cells develop at sites of inflammation as part of the inflammatory immune response (Burkitt et al., 1997) and are present in human cell culture after the fusion of monocytes following exposure to lymphokines (cytokines produced by lymphocytes) (Baskar et al., 1994). The ability to culture these cells from marsupial blood provides an opportunity for future studies of their capacity to respond to bacterial invasion and to investigate secretory products involved in the formation of granulomas, since monocyte-derived cytokines are involved in the formation of granulomas in eutherian mammals (Agostini et al., 1998).

Cell forms cultured from monocytes in tammar wallaby blood also included cells resembling plasmacytoid monocytes that are monocyte-derived cells of the dendritic cell type. These cells may be found in human blood and lymph nodes (Cella et al., 1999) and play a role in inflammatory reactions where they secrete relatively large quantities of Type 1 interferons. Cell types also present in culture included those with many 'finger-like' projections, which serve to increase the available surface area for antigen uptake and contact with T cells, and amorphous cell structures that resembled
dendritic cells isolated from the peripheral blood of humans (O’Doherty et al., 1994), cows (Renjifo et al., 1997), rats (Kudo et al., 1997) and horses (Siedek et al., 1997).

In human blood, dendritic cells comprise two distinct subsets; an immature bone-marrow derived population that travel to skin or other non-lymphoid organs and a second population that travels directly to lymphoid organs prior to activation by antigen. These cells play an important role in antigen presentation and are essential in the generation of a primary MLC response (O’Doherty et al., 1994; Siedek et al., 1997). Given the reported lack of a definitive MLC response in marsupials (see 1.5.6.1) and the potential role of dendritic cells in cell-mediated responses, it is important to characterise the function of these cells in marsupials. The development of the culture system described in this study will clearly facilitate this future research.

Co-culture of lymphocytes or addition of lymphocyte conditioned media was necessary to promote tammar wallaby monocyte transformation and to sustain the viability of the adherent cell population. This requirement for lymphocytes or media containing lymphocyte or monocyte secreted factors, suggests that growth factors or cytokines were required for the development of tammar wallaby adherent cells. Within the LCSFM culture system, the transformation of adherent cells into macrophage and dendritic cell forms occurred over a shorter time interval when these cells were in the presence of bacteria or dead and dying lymphocytes. This suggests that a phagocytic stimulus may be necessary to initiate activation of these cell forms in culture. This observation is similar to a description of the transformation of non-dividing human monocytes into dendritic cells where it was suggested that phagocytic stimuli, including the ingestion of apoptotic cells and exposure to micro-organisms, may be a maturation stimulus for these cells (Randolph et al., 1998). The ability of Con A-activated monocytes to remain viable and to proliferate after mitogen stimulation also confirms the necessity for these cells to be activated before they continue to grow in culture.

There are currently no reports of the functional immune capacity of marsupial peripheral blood monocytes. However, there have been a small number of studies that describe the functional behaviour of marsupial macrophages, all in relation to the role of these cells in response to mycobacterial infection. In one study by Moriarty
and Thomas (1986), the chemotactic properties of peritoneal macrophages (PM) from the brushtail possum were found to be different from those of a eutherian mammal, the guinea pig. Possum PM were non-responsive when exposed to a chemotactic factor produced by mitogen treatment of splenic macrophages which was in contrast to the response of guinea pig PM that responded to this chemotactin with an increase in their migration rate. The lack of response by possum macrophages was thought to contribute to the poor granuloma formation observed in possums infected with mycobacterial agents. However, similar to responses of macrophages from the guinea pig, and other species such as rats and humans, possum alveolar macrophages share the ability to suppress the responses of lymphocytes to mitogens and antigens (Buddle and Young, 2000). It is not yet known whether tammar wallaby macrophages share these properties. This is, in fact, the first study to characterise some of the immunological defence properties of these cells.

Despite the low numbers of monocytes recovered in this study, cells cultured using optimum conditions retained their viability in on-going culture. To avoid further loss of cells and damage to their \textit{in vitro} functionality, the continued viability of these cells was assessed using the MTT assay. For applications such as the determination of adherent cell viability, this assay has the advantage of staining cells \textit{in situ}, which also allows for their concurrent enumeration. Although trypan blue staining is routinely used for viability assessment in most cell culture experiments (Brousseau \textit{et al.}, 1999), a number of studies of human adherent cells such as blood monocytes (Epstein \textit{et al.}, 1991) and a macrophage bone marrow cell line (Shi \textit{et al.}, 1993) have also applied the MTT assay for this purpose.

Once monocytes and granulocytes were successfully isolated from the peripheral blood of the tammar wallaby, an assessment of the phagocytic and antimicrobial responses of these cells was undertaken.

\textbf{The Phagocytic Response}

In most mammals studied to date, the phagocytosis of antigens \textit{in vivo} consists of a number of events that are necessary for the effective ingestion and ultimate destruction of unwanted material. Cells must first recognise (polarisation) and then mobilise (chemotaxis) toward the site of injury or infection before they ultimately
ingest the pathogen in question (phagocytosis). Once inside the phagocytic cell, a number of biochemical agents are released that are toxic to most micro-organisms and cause their destruction (see 1.5.4.1.3). Phagocytic cells also possess a number of non-oxidative defence strategies that may deal directly with invading bacteria. In this study of tammar wallaby phagocytes, similar assays were performed on both monocytes and granulocytes to assess these innate defences, but due to limited cell numbers, only the more active and readily isolated granulocyte populations were studied for their ability to respond to chemotactic agents.

**Polarisation Responses**

A number of techniques have been used to assess leukocyte responses to chemotactic factors, particularly those of polymorphonuclear cells (reviewed by Haddox and Pfister, 1993). In these assays, cells are exposed to uniform concentrations of stimulating factors that are assessed for their ability to promote a change in the cytoskeletal arrangement of cells that occurs prior to locomotion (Haston and Wilkinson, 1988). These factors can then be screened for their ability to promote chemotaxis in the cell population under study (Shields and Haston, 1985).

After exposure to a variety of stimulating agents, the morphology of polarised granulocytes from the tammar wallaby was similar to that of human cells cultured under similar conditions. Approximately 95% of tammar wallaby peripheral blood granulocytes remained spherical when incubated in control solutions of HBSS/MOPS, which is consistent with results for control cells in human studies (Haston and Wilkinson, 1988). However, as mentioned previously, storage on ice appeared to pre-activate cells in some assays, which reduced this control figure to approximately 70%. It has been suggested that this pre-activation is a result of the disassembly of microtubules within granulocytes which leads to physical changes that mimic polarisation responses (Haston and Wilkinson, 1988) although this theory is not commonly supported (see Bignold, 1992). Whatever the underlying mechanism for these responses, the waves of contraction that cause the changes in cellular morphology in suspension are translated to movement when the cell meets a substratum. Thus, there is a correlation between the ability of a substance to effect polarisation and its ability to promote chemotaxis (Wilkinson and Haston, 1988). When tammar wallaby granulocytes were exposed to a variety of potential polarising
agents in solution, these cells changed from a smooth, spherical shape to cells with
ruffled cytoplasm that possessed singular or numerous cell extensions. In response to
some treatment agents, elongated cell bodies were apparent, with either blunt or
narrow extended pseudopods. These responses are all similar to those produced by
human granulocytes, where the various shapes represent transitional cell forms
between the resting, unstimulated cell and the highly activated, polarised cell
(Bignold, 1992).

When tammar wallaby granulocytes were exposed to serum, greater than 80% of cells
adopted polarised morphology. This effect is most likely the result of exposure to
serum components such as complement factor C5a and serum albumin which are
known chemotactic agents for eutherian cells (Falk et al., 1980; Stickel et al., 1985;
Bray, 1992). Similar polarisation results were obtained for cell treatments with
supernatants recovered from both gram positive (S. aureus) and gram negative (E.
coli) bacterial cultures. These polarity affects were mirrored in the chemotaxis
responses obtained for these stimulants (see later discussion). Bacterial LPS also
promoted polarisation responses in granulocytes, although the degree of cytoskeletal
rearrangement appeared to be related to the concentration of LPS. This dose-response
effect was also obtained for oxidative burst responses (see later discussion), which
confirms that the polarisation assay is useful for assessment of cell activation.

The variability of response to the chemotactic peptide fMLP was reflected across
results from polarisation, chemotaxis and oxidative burst assays undertaken in this
study. This variability is also found in fMLP responses of cells from domestic
mammals such as the dog and horse (Stickel et al., 1985) but is different to that of
human and guinea pig cells that display a strong response to this peptide (Shields and
Haston, 1985). Human neutrophils rapidly become polarised in solution in response to
uniform concentrations of fMLP as evidenced by the appearance of a leading edge
and tail. In contrast, lack of fMLP responsiveness has been reported for granulocytes
from the dog, pig, horse and cow (see Stickel et al., 1985). This non-responsiveness
has been linked to decreased numbers of cell surface receptors on the granulocytes of
these cells that recognise fMLP. Results from the present study of tammar wallaby
granulocyte responses to fMLP suggest these cells possess fMLP receptors
intermediate in numbers between those of humans and a range of domestic species
(see earlier discussion). This suggestion may be confirmed by quantitation of fMLP receptors by radiolabelling techniques (Stickle et al., 1985) and should provide useful information regarding the ability of tammar granulocytes to respond to infection by bacteria that produce these factors.

*Chemotactic Responses*

The chemotactic behaviour of granulocytes can be assessed by inspection of the migration of these cells on a physical substrate after treatment with stimulating factors that are identified by polarisation studies (Haston and Shields, 1985). In this study of tammar granulocytes, two methods were used to assess the chemotactic responses of these cells; the under-agarose method and a modified chamber technique.

Consistent with the polarisation responses also investigated in this study, results from chemotaxis assays identified that serum, yeast-activated serum and bacterial products were chemotactic for tammar wallaby granulocytes. However, whilst some of the test agents were chemotactic in tammar wallaby and other mammalian species, there were some differences observed between these responses. Similar to the polarisation response for fMLP, the chemotactic response for this peptide was variable for tammar cells and therefore appears to be intermediate between the response of human neutrophils (Bass et al., 1978) and those of other species such as the dog (Stickle et al., 1985).

Although a full set of dose-response experiments was not undertaken on the treatment agents applied to granulocytes in this study, it appears that there is a concentration-dependent relationship exhibited by granulocytes to both soluble and particulate agents. In polarisation and chemotaxis studies, 10μg/mL of LPS caused measurably higher responses than 100μg/mL LPS. This is different from the response of human cells where intradermal injection of LPS results in the accumulation of neutrophils *in vivo* but where LPS is not chemotactic *in vitro* at any concentration (Yoshimura et al., 1987). In some species such as the dog, the LPS response is activated by the presence of serum (Chammas and Hagiwara, 1998) but this was not a requirement for LPS stimulation of tammar cells.
Bacteria and supernatants of cultured bacteria were chemoattractive for *M. eugenii* granulocytes, which is consistent with the response of horse neutrophils when treated with culture supernatants from *Streptococcus faecalis* (Blancquaert et al., 1988). Similarly, serum and serum-derived test agents also promoted strong chemoattractive responses in tammar wallaby granulocytes. Zymason-activated serum is known to stimulate chemotaxis in both human and pig neutrophils (Bass et al., 1978; Goodman et al., 1991) and this response is also enhanced when serum is added to the agarose matrix in chemotaxis under-agarose assays for horse, human (Blancquaert et al., 1989) and dog neutrophils (Petersen et al., 1998).

As is the case with polarisation effects, the ability of a cell to respond to a chemoattractant relies on the cell possessing a plasma membrane receptor that recognises the chemoattractant under test (Harvath et al., 1994). Results from this study therefore suggest that tammar granulocytes appear to possess a similar array of cell surface receptors capable of recognising immunostimulatory molecules when compared with other mammals studied to date.

*Phagocytosis*

In this study, tammar wallaby phagocytes demonstrated the capacity to ingest both inert and biological particles with or without the presence of serum. Larger numbers of smaller test particles such as 1.1μm latex beads and bacteria were ingested by monocytes and granulocytes. The larger yeast particles and 3μm latex beads were ingested in lower numbers, which appeared to be related more to the total volume of the ingesting cells rather than their phagocytic capacity. These findings are consistent with those for macrophages from eutherian mammals that are also able to ingest foreign particles without the need for opsonisation (Lehnert and Tech, 1985). However, ingested particle numbers rose when cells were cultured in the presence of serum or when phagocytic particles were pre-opsonised with serum, except when test agents were added directly to whole blood. The role of serum in increasing the numbers of particles ingested by monocytes and granulocytes suggests a role for as yet unidentified serum components in the phagocytic processes of tammar wallaby cells.
Adherent cells, especially those with dendritic morphology, appeared to become 
stimulated when co-cultured with bacteria and were observed to phagocytose these 
cells. Similarly, cells within adherent cell cultures ingested dead and dying 
lymphocytes after co-incubation for at least 3 days. Both bacteria and apoptotic cells 
are known to stimulate the phagocytic processes in eutherian leukocytes (Randolph et 
al., 1998), which confirms the similarity of function of these cells to their eutherian 
counterparts.

In summary, tammar wallaby monocytes and granulocytes demonstrated the capacity 
to recognise, migrate towards and ingest biological agents such as yeast and bacteria 
when assessed using polarisation, chemotaxis and phagocytosis assays modified from 
those used on eutherian cells. Polarisation and chemotaxis was also induced in these 
cells after stimulation with a range of biological materials such as LPS and serum. 
Monocytes and granulocytes demonstrated phagocytosis of both inert and biological 
particles in the presence or absence of serum, although increased phagocytosis was 
apparent when serum was used as a particle opsonin or when added to the culture 
medium.

Oxidative Responses

In this study, tammar wallaby phagocytic cells mounted oxidative burst responses to a 
number of soluble and particulate agents. These responses were consistent with those 
of eutherian cell populations in that neutrophils appeared to produce increased levels 
of ROIs when compared with monocytes or macrophages (Goldsby et al., 2000).

Monocytes

Similar to the relative responses of eutherian phagocytes, the respiratory burst stimuli 
of tammar wallaby monocytes was of a lower magnitude when compared with 
granulocytes cultured under similar conditions (Beaman and Beaman, 1984). These 
cells produced detectable superoxide responses after treatment with PMA, although 
cell numbers were low and the level of response was difficult to quantify.

Macrophages from a eutherian mammal, the deer, also produce oxidative responses 
after treatment with the phorbol ester, PMA (Cross et al., 1996). However, the 
magnitude of this response occurs at the lower limit of NBT detection in the present 
study, which suggests that the NBT assay may not be suitable for the quantitation of
monocyte or macrophage responses unless these cell types are present in greater numbers than was used in the current study.

The results of the monocyte oxidative assays, together with the visual identification of NBT formazan products within stimulated adherent cells, indicate that oxidative burst products are produced by tammar wallaby monocytes. However, for quantitation of these results, increased cell numbers and longer incubation times are required to increase responses to detectable levels.

**Granulocytes**

Inert and biological particles, endotoxin (LPS) and phorbol myristate acetate (PMA) are known to stimulate the respiratory burst of human leukocytes (Root and Cohen, 1981). Results obtained from tammar wallaby granulocytes after stimulation with these agents suggests that they also stimulate the respiratory burst response in granulocytes obtained from the peripheral blood of this marsupial.

In this study, the reduction of NBT to NBT formazan was used to localise (Pick, 1986) and quantify (Rook et al., 1985) the degree of production of superoxide anion within tammar wallaby cells after stimulation with both PMA and LPS. The positive NBT responses obtained for tammar wallaby granulocytes were indicative of functional NADPH oxidase activity (Baehner et al., 1975) within these cells and are consistent with responses obtained for normal functional granulocytes from human subjects (Repine et al., 1979). In NBT slide assays, the oxidative response was accompanied by a visible increase in cell size and obvious degranulation, events which precede phagocytosis in activated cells (Morel et al., 1991). In control NBT slides, while the majority of cells were NBT negative, in many of the samples up to 10% of unstimulated cells were NBT positive. This response may reflect a small degree of activation associated with the adherence of cells to the glass substratum. Adherence to endothelial cells and the extracellular matrix *in vivo* occurs after cells have been stimulated by a chemotaxin, a process that occurs prior to phagocytosis and generation of the oxidative burst (Petersen et al., 1999).

In LPS stimulated cells in the NBT slide assay, between 60 and 85% of granulocytes were NBT positive when incubated for 60mins. This response is less than that
reported for human cells where 90% of granulocytes are NBT positive using this assay (Repine et al., 1979). However, human NBT granulocyte assessments are usually undertaken in the presence of serum since human neutrophils, like horse neutrophils, do not produce direct LPS oxidative burst products unless serum factors are present (Repine et al., 1979; Benbarek et al., 1998). These results suggest that tammar wallaby granulocytes are more sensitive to direct stimulation with endotoxin since cells producing oxidative responses, whilst present in lower numbers, produced these responses in serum-free media.

Values for resting, unstimulated NBT-treated granulocytes in the NBT quantitative assay varied between 3.2μg/10⁶cells and 283.5μg/10⁵cells (mean = 77.1μg, SD = 91μg). A human sample tested for comparative purposes, also fell within this range. The very large standard deviation appeared to reflect the differences between and within animals as well as the different levels of pre-activation across the range of isolation procedures employed in this study. This variation was standardised by expressing the values for treated versus untreated cells as the Respiratory Burst Index (RBI). Whilst most responses achieved using the NBT assay were consistent with those of eutherian phagocytic cells (Root and Cohen, 1981), there were variable responses to fMLP, which mirrored the polarisation and chemotaxis results from prior experiments. Tammar wallaby granulocytes demonstrated an increased NBT response to serum, serum-opsonised zymason and zymason in the presence of serum. Zymason and serum stimuli also cause increases in the NBT responses of bovine granulocytes (Roth and Kaeberle, 1981).

Variation in oxidative responses appeared to be related to the type of assay used to measure these responses. Oxidative species generated after PMA stimulation in the Cytochrome C assay were quenched by superoxide dismutase, which indicates the specificity of this reaction for the production of superoxide anions. In contrast, LPS did not produce superoxide within the 10 min incubation time in the Cytochrome C assay but did produce a significant (P<0.05) response when treated with LPS and cultured with NBT for longer periods of 30 and 60mins. These responses suggest one of two outcomes. The first is that the products detected by the NBT assay include other oxidative species such as hydrogen peroxide that are not measured by the Cytochrome C assay. Secondly, and more probable, is that the two assays are
discriminating between the intracellular and extracellular pools of superoxide anions found within the cells and in the extracellular medium after respiratory burst stimulation (Pick, 1986). Results from this study therefore suggest that of the two cell stimulants, PMA treatment causes a faster cellular response since superoxide appears to be released quickly into the external environment whereas oxidative species generated after stimulation with LPS remain localised within the cell. The results for the stimulation of tammar wallaby granulocytes with LPS and PMA using the Cytochrome C assay are consistent with the results obtained for equine leukocytes (containing 65% polymorphonuclear cells and 25% mononuclear cells) where LPS at both 1 µg/mL and 5 µg/mL did not cause measurable superoxide anion production within a two hour incubation period. Also consistent is that treatment with PMA at 0.5 µg/mL caused a significant increase in production of this anion within 15 mins of incubation (Seethanathan et al., 1990).

In this study, the phagocytosis of both inert and biological particles by tammar wallaby granulocytes was accompanied by an oxidative burst as assessed by the NBT response, with a strong arc of NBT depositing near the particle being ingested. This is consistent with findings in granulocytes from eutherian mammals such as the dog (Shearer and Day, 1997) and in bovid granulocytes where the phagocytosis of serum-opsonised zymosan caused a significant increase in the NBT response compared with zymosan alone (Roth and Kaeberle, 1981).

Serum components appear to play a dominant role in the nature of the phagocytic response and variations in responses to phagocytic stimuli may be due to the differences in serum components found between species. In one study of human polymorphonuclear cells (PMN), serum complement was necessary for the detection of LPS mediated chemiluminescent responses (Nicotra et al., 1985) and in another report of bovid granulocyte responses, conglutins present in serum were found to interfere with the ingestion of zymosan (Roth and Kaeberle, 1981). Clearly, the nature of serum components and their role in regulating the phagocytic responses of marsupials needs further clarification. Results from this study suggest that there are significant differences in the requirement for serum in the generation of toxic oxygen species in tammar granulocytes compared with those of other mammals, while there are similarities in the role of serum as an opsonin in phagocytic responses.
Nitric Oxide

Monocytes

As part of the arsenal of biochemical microbicidal molecules available to the host, NO influences both resistance and susceptibility to disease (Ghigo et al., 2001; Mohan et al., 2001) although the bactericidal properties of this compound remain controversial (Zhao et al., 1997; Carpenter et al., 1998). Specifically, this molecular species is known to play a role in the pathogenicity of intracellular pathogens such as *T. gondii* (Taylor-Robinson, 1997) and *M. avium* (Nathan and Hibbs, 1991). In the present study of tammar wallaby monocytes, an increase of approximately 250nmol of NO by between $1 \times 10^5$ and $5 \times 10^5$ cells was produced in response to LPS at 1µg/mL. It is difficult to directly compare this level of NO production with that of other species since low numbers of cells were used in these assays. However, even at this low cell number it was apparent that NO was produced by tammar monocytes as a response to treatment with LPS. The production of NO by monocytes from eutherian mammals is variable and appears to depend on the particular species and the nature of the stimulant. Deer monocytes failed to produce NO when stimulated with 10µg/mL LPS, when under the same conditions, mouse monocytes produced 1.5-2.5µmol nitrite per $10^5$ cells (Cross et al., 1996). Bovine monocytes produced an increase of 0.8µM NO in response to LPS at 10µg/mL (Barbuddhe et al., 1998) and up to 15µM NO in response to treatment with BCG and IFN (Carpenter et al., 1998). Further studies with increased numbers of monocytes and a broader range of NO triggering agents would further clarify this response by tammar monocytes and help to define the nature of the biochemical immune defence mechanisms available to these cells.

Although only a single set of experiments were performed to detect the capacity of tammar wallaby granulocytes to express NO, results suggest that this molecular species is produced by this cell population. Levels of NO equalling that produced by stimulated eutherian macrophages (see above) were constitutively produced by the granulocytes of one animal and this further increased in response to stimulation by PMA. These results should be further characterised since the relatively high levels of NO produced by control cells in this study may be an artefact of culture or may indeed represent a dominant role for NO in the innate immune defence system of these animals.
Application of this assay would be useful to determine the nature of the cellular response to antigen in the case of disease investigation of macropodid species. For example, in the present study, monocytes from most animals tested generated a superoxide burst when treated with PMA, but did not produce nitrite in response to this agent. These responses were reversed in monocytes from animal 1994, where cells from this animal responded to PMA with the production of nitrite but not superoxide. This differential response may be significant in the susceptibility of this animal to pathogens that are destroyed by generation of superoxide radicals or conversely, may afford a protective response to pathogens that are susceptible to exposure to NO.

**Non-oxidative Responses**

Mammalian neutrophil granules contain a variety of antimicrobial effector molecules that may enter the phagosomes or the cell exterior via degranulation (Gudmundsson and Agerberth, 1999). In this study, tammar wallaby neutrophils contained a number of different granule populations that were clearly visible in electron micrographs of these cells (see Chapter 3). To investigate the antimicrobial activity of compounds extracted from these granules, acid-extractable components were isolated from the granules of a tammar wallaby PMC population isolated from peripheral blood.

Granules extracted from neutrophils and eosinophils contained proteins that exhibited antimicrobial activity when co-cultured with *S. aureus* and *E. coli*. Fraction 3 (<3kDa) most likely contained proteins up to 10kDa since effective exclusion of proteins with molecular weights greater than those of the molecular weight cut-off filters only occurs when the membrane pore size is 3 to 6 times smaller than that required. Thus, this fraction may have contained defensin molecules, since these smaller antibacterial peptides are known to discriminate between gram positive and gram negative bacteria (Gennaro *et al.*, 1983), similar to the findings in the present study. Fraction 1 (50K-3K granule extract) would also be expected to contain some of these molecules as well as other higher molecular weight fractions.

Antimicrobial granule activity has been identified in bovine granulocytes and molecules with molecular weights of 37 000, 54 000, 57 000 and 90 000 were implicated in these activities (Gennaro *et al.*, 1983). The 90 000 MW molecule was
identified as lactoferrin, but the other molecules were not identified. All of these bovine-granule derived molecules targeted *E. coli* in the bactericidal assays performed in this work and are consistent with the findings for tammar wallaby granule activities (see Table 4.7).

Myeloperoxidase (MPO) was also most likely present in the granules extracted from tammar wallaby granulocytes, since the colour of the granule preparations was pale green, which is consistent with the presence of heme proteins in these preparations. Furthermore, the peroxidase enzyme was identified in tammar wallaby phagocytes when assessed using DAB testing (see Figure 3.12). MPO is a constituent of the oxygen-dependent microbicidal defence portfolio of granulocytes found in the azurophil or primary granules (Root and Cohen, 1981). However, the importance of MPO is unclear in eutherian phagocytic cells since human subjects with cells deficient in MPO are no more susceptible to bacterial infections than normal subjects (Beaman and Beaman, 1984). MPO deficiency can be an acquired disorder as well as inherited and may be caused by such factors as deficiencies in antibody and complement proteins as well as pharmaceuticals such as corticosteroids (Haen, 1995).

Other molecules that may have been responsible for the antimicrobial properties of neutrophil granules include proteases such as elastase, Cathepsin G and Proteinase 3. These enzymes are most effective at neutral pH, an environment that was used for culture in the present study. Protease inhibitors were not added to the isolated granule fractions in the current study so that these, and other molecules found in eosinophil granules that are known to be proteolytic (Weller, 1994), could exert their maximal effects. Bactericidal/Permeability-Increasing Protein (BPI) is another antimicrobial substance associated with azurophilic granule membranes of myeloid cells (Egesten et al., 1994) that is produced exclusively by neutrophils and has both bacteriostatic and bactericidal effects on gram-negative bacteria (Weiss and Olsson, 1987).

Human eosinophilic granules do not contain lysosome, cationic proteins or lactoferrin (Root and Cohen, 1981) and are principally involved in the destruction of the cell membranes of metazoan parasites by antibody-mediated degranulation of major basic protein and arylsulfatases. The eosinophil component of the granulocytes tested in this study comprised up to 10% of the total granulocytes, so it is possible that
eosinophilic granule contents contributed to the bactericidal/bacteriostatic properties described in this study.

Whilst the nature of the antibacterial species present within the granules of tammar wallaby neutrophils and eosinophils remains unidentified, results from this study confirm the existence of low molecular weight species within these granules that are cytotoxic to gram-positive bacteria and those of a higher molecular weight that are bactericidal for gram-negative bacteria.

4.4 Conclusion

In this study, tammar wallaby phagocytes were successfully isolated using methods adapted from those used for the isolation of similar cell populations in eutherian mammals. These cells demonstrated the capacity to respond to soluble and particulate stimuli by undergoing phagocytosis and mounting both oxidative and non-oxidative defences. This is the first report identifying marsupial phagocytic populations and results from this study confirm the commonly held belief that both monocyte and granulocyte peripheral blood cells are able to mount innate immune defence responses similar to those of eutherian mammals.
CHAPTER FIVE

The Capacity of Peripheral Blood Mononuclear Cells to Generate Responses In Vitro

5.1 Introduction

In this study, assessment of the capacity of tammar wallaby peripheral blood mononuclear cells (PBMC) to mount in vitro responses was undertaken in three separate stages.

1. Experimental conditions were optimised for the assessment of the capacity of tammar wallaby PBMC to respond to polyclonal activation using plant lectins.

2. The ability of activated marsupial lymphocytes to secrete immunomodulatory molecules (cytokines) using these optimised conditions was verified and

3. The expression and secretion of cytokine molecules by activated cells in culture was documented.

5.1.1 Mitogens

Phytohaemagglutinin (PHA), Concanavalin A (Con A) and Pokeweed Mitogen (PWM) are non-specific stimulants that activate the immune cells of a variety of animals without the need for prior antigen exposure (Janossy and Greaves, 1971; Kristensen et al. 1982b). When exposed to these compounds in vitro, lymphocytes from eutherian mammals show morphological changes that mimic the changes that occur in cells treated with specific antigens (Sharon, 1983). Plant lectins such as those mentioned above, vary in their ability to stimulate B cells, T cells or both lymphocyte populations because they recognise and bind to carbohydrate residues of different glycoproteins on cell surfaces that are thought to be unique for different cell populations (Tizard, 2000). The binding and cross-linking of these molecules leads to agglutination of activated cells and proliferation of the target cell population (Rose et al., 1992). Similarly, products derived from bacterial cells, such as bacterial LPS, may also cause stimulation of lymphocyte populations.
5.1.1.1 Phytohaemagglutinin (PHA)

PHA is a multivalent lectin that binds to cell surface glycoproteins of eutherian T lymphocytes (Sharon, 1983). It is extracted from red kidney beans (*Phaseolus vulgaris*) and has a molecular weight (MW) of 120 000Da. This lectin specifically recognises the N-acetyl-galactosamine sugar moiety, which is found in association with the CD3 complex and the T cell receptor. PHA is known to stimulate both CD4\(^+\) and CD8\(^+\) T cells (Rose *et al.*, 1992; Wang *et al.*, 1997; Tizard, 2000).

5.1.1.2 Concanavalin A (Con A)

Con A is a plant lectin derived from Jack beans (*Canavalia ensiformis*). It has a MW of 108 000Da and a different carbohydrate specificity to PHA, recognising α-D-mannose and α-D-glucose when they are present on the surface of eutherian lymphocytes (Cuatrecasas and Tell, 1973; Tizard, 2000). Con A is also a T cell mitogen (Sharon, 1983). However, in contrast to PHA, it induces a high level of activation of CD8\(^+\) cells in mammals (Wang *et al.*, 1997).

5.1.1.3 Pokeweed Mitogen (PWM)

The plant lectin Pokeweed mitogen (*Phytolacca americana*) has specificity for the sugar di-N-acetylchitobiose (Sharon, 1983). It has a MW of 32 000Da, and in eutherian cell culture, stimulates predominantly CD4\(^+\) cells. A low proportion of CD8\(^+\) cells are also stimulated by this mitogen, which is thought to reflect the activation of T-helper cells in inducing immunoglobulin production (Wang *et al.*, 1997). Exposure to PWM stimulates T cell dependent responses.

5.1.1.4 Phorbol Myristate Acetate (PMA) and Ionomycin

PMA is a protein kinase C (PKC) activator (Rose *et al.*, 1992). In mammalian cell culture systems, it is used as a diacylglycerol analogue and co-mitogen with the anti-CD3 antibody. The combination of PMA with the calcium ionophore, Ionomycin, activates the pathway distal to the CD3 receptor. Ionomycin induces production of inositol trisphosphate and usually results in production of IL-2 and expression of the IL-2 receptor (Geha and Chatila, 1992).
5.1.1.5 Lipopolysaccharide (LPS)
Mammalian responses to gram-negative bacteria generally arise due to interaction with the LPS components of the bacterial cell membrane (Borregaard et al., 2000). Thus, the lipid portion of LPS is used as a stimulant of in vitro mitogenic activity. Unlike PHA, Con A and PWM, LPS preferentially activates the B lymphocytes of eutherian mammals.

5.1.2 Mitogen Stimulation of Marsupial Cells
Marsupial cells from a range of animals have previously been shown to respond to mitogen stimulation in vitro using a variety of culture conditions, mitogens, mitogen levels and incubation times (see 1.5.6.2.2 and Table 5.1). However, these responses were varied and the proliferation experiments from which they arose were limited to those undertaken in culture media supplemented with foetal calf or autologous serum and where proliferation values were determined using the tritiated thymidine uptake technique. To date, there has only been a single study of tammar wallaby leukocyte proliferation, which used quokka serum as the culture supplement (Ashman et al., 1976). Under these culture conditions, tammar wallaby lymphocyte proliferation responses were poor. Given the status of this animal as a model macropod, it is important to investigate this apparent deficiency and to gather baseline information regarding the ability of PBMCs from this species to mount an immune response.

5.1.3 Marsupial Cytokines
The identification of marsupial cytokines to date has relied solely on the comparison of cDNA sequences amplified from marsupial tissues, with known cytokine genes from other species (reviewed by Harrison and Wedlock, 2000). The cytokines detected thus far using this RT-PCR approach include TNF-α from both the brushtail possum (Wedlock et al., 1996) and the tammar wallaby (Harrison et al., 1999), IL-10 (Wedlock et al., 1998), IL-1β (Wedlock et al., 1999b) and Leukemia Inhibitory Factor (Cui and Selwood, 2000) from the brushtail possum, and IL-5 (Hawken et al., 1999), Lymphotoxin-α and β (Harrison and Deane, 1999a and b) and Type I IFNs (Harrison et al., 2002) also from the tammar wallaby.

TNF-α, IL-1β and IL-10 are cytokine molecules that are strongly implicated in the control of inflammatory responses (Lowry, 1993) and immune responses to
intracellular pathogens (Tsukaguchi et al., 1999). These molecules are also expressed \textit{in vitro} in response to polyclonal activators. Since the sequence for tammar wallaby TNF-\(\alpha\) has previously been reported (Harrison et al., 1999), the present study sought to identify both IL-1\(\beta\) and IL-10 from mitogen activated cells of this species in order to facilitate further studies into the regulation of the marsupial immune response.

\subsection*{5.1.3.1 IL-1\(\beta\)}
There are two different forms of IL-1; IL-1\(\alpha\) and IL-1\(\beta\). These molecules are produced by different genes and share less than 30\% structural homology (Dinarello, 1992). IL-1 is synthesised by LPS or TNF-\(\alpha\) activated mononuclear phagocytes and also by both endothelial and epithelial cells. Like TNF-\(\alpha\), one of the primary functions of IL-1 is to control the host response to inflammation. IL-1\(\beta\) was first reported in marsupials in a functional and electrophoretic study of the supernatants of LPS-treated macrophages in the South American Opossum (Brozek and Ley, 1991). Later, cDNA for this cytokine was characterised by Wedlock et al. (1999b), who detected this molecule in LPS-treated brushtail possum alveolar macrophages.

\subsection*{5.1.3.2 IL-10}
In eutherian mammals such as humans and mice, IL-10 is a negative regulator of the immune response (Murray et al., 1997). It is secreted by a number of different cell types including T and B lymphocytes, monocytes, macrophages and keratinocytes. The primary roles of IL-10 are to inhibit macrophage production of cytokines such as TNF-\(\alpha\) and IL-1 and to limit the ability of macrophages to act as accessory cells in T cell activation by reduction of MHC Class II and co-stimulator molecules (Bejarno et al., 1992). Counter-regulatory cytokines such as IL-10 are also produced in normal host immune responses in order to control the over-production of immunostimulatory molecules such as IFN-\(\gamma\) and TNF (Moore et al., 2001). The identification of molecules associated with immunoregulatory functions is therefore a necessary facet of investigations into disease susceptibility. Thus, the detection of IL-1\(\beta\) and IL-10 in the model species, the tammar wallaby, was a preliminary step in the investigation of these molecules in endangered macropod species.
<table>
<thead>
<tr>
<th>Species</th>
<th>Mitogen</th>
<th>Range of mitogen tested</th>
<th>Optimum mitogen concentration</th>
<th>Cell Preparation</th>
<th>Cell density</th>
<th>Media</th>
<th>CPM</th>
<th>Other</th>
<th>SI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. virginiana</em></td>
<td>PHA-M</td>
<td>1:2.5-1:100 dilutions</td>
<td>1:10</td>
<td>sedimentation (Boyum)</td>
<td>2.5x10⁴/2.5mL</td>
<td>EMEM+20% FCS</td>
<td>72hrs, 0.5µCi (12Ci/mumole) for 5hrs, control: 604, PHA: 6660</td>
<td>Viability: 65-75% (3days), 40-50% (5 days), 20-25% (7 days)</td>
<td>Prasad et al., 1971</td>
<td></td>
</tr>
<tr>
<td><em>T. vulpecula</em></td>
<td>PHA</td>
<td>0.4, 2, 10, 50µg/mL</td>
<td>50µg/mL</td>
<td>spleen cells</td>
<td>1 x 10⁶ cells/mL</td>
<td>EMEM+10% FCS</td>
<td>(x10⁷/x10⁶ cells) control: 1:1-4:6 test: 13.9-54.6 4hrs after culture time with 1.5µg tritium</td>
<td>Viability: 65-75% (3days), 40-50% (5 days), 20-25% (7 days)</td>
<td>Moriarty, 1973</td>
<td></td>
</tr>
<tr>
<td><em>S. brachyrurus</em></td>
<td>PHA-P, Con A, PWM</td>
<td>not reported</td>
<td>60µg/mL for both antigens. Con A 25µg/mL</td>
<td>buffy coat + RBC lysis</td>
<td>1 x 10⁶ cells/mL in 200µL</td>
<td>RPMI 1640 + 10% FBS+2mM glutamine</td>
<td>72hrs, 1µCi ³H (5Ci/mmol) for 18hrs</td>
<td>EDTA low SI to Con A reflected in greatest SI of 2 for one animal</td>
<td>From 3 - 22 as cpm stim/ cpm control</td>
<td>Buddle et al., 1994</td>
</tr>
<tr>
<td><em>M. eugeniae</em></td>
<td>PHA-P, Con A, PWM</td>
<td>µg PHA: 1.25, 2.5, 5, 12.5, 25 µg Con A: 2, 5, 10, 20, 50, 100 µg PWM: 6.6, 33.3, 66.6</td>
<td>PHA: 2.5-5µg Con A: 5µg PWM: 33.3µg</td>
<td>dextran sedimentation</td>
<td>2.5 x 10⁷ mononuclear cells/mL in 0.2mL</td>
<td>McCoy's + 5% HI homologous quokka serum</td>
<td>dpm(from graph) PHA: 75000 Con A: 5300 PWM: 5200 0.5µCi ³H/well at 24hrs. Left for 24 hrs.</td>
<td>heparin all data from graph Viability dropped from 90% to 50% @ 5 days</td>
<td>Ashman et al., 1976</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

212
<table>
<thead>
<tr>
<th>Species</th>
<th>Mitogen</th>
<th>Range of mitogen tested</th>
<th>Optimum mitogen concentration</th>
<th>Cell Preparation</th>
<th>Cell density</th>
<th>Media</th>
<th>CPM</th>
<th>Other</th>
<th>SI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. domestica</em></td>
<td>PHA-P, Con A, PWM</td>
<td>range not stated</td>
<td>Con A: 25μg/mL</td>
<td>ficoll gradient</td>
<td>10^5 cells/well in 200μL</td>
<td>RPMI + 10% FCS</td>
<td>Con A: 26575 1μCi 3H/well at 72hrs. Left for 18 hrs. Mean control values: 371 cpm</td>
<td>ACD or Aisever's</td>
<td>19.4</td>
<td>Infante <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><em>P. cinereus</em></td>
<td>PHA-P, Con A PWM</td>
<td>PHA-P &amp; ConA: 0.5 - 10μg/mL, PWM: 0.5-100μg/mL</td>
<td>PHA: 1-6μg/mL, ConA: 1-6μg/mL, PWM: 50μg/mL</td>
<td>ficoll gradient</td>
<td>1.8x10^5 cells/well in 200μL</td>
<td>RPMI + 5% FCS</td>
<td>PHA: 10590-123585, Con A: 22 750-140 825, PWM: 30 310-148 900, 0.4μCi 3H/well at 72-96hrs for 16hrs.</td>
<td>PHA: 335-488, Con A: 15-396</td>
<td>Wilkinson <em>et al.</em>, 1992b</td>
<td></td>
</tr>
<tr>
<td><em>D. matschiei</em></td>
<td>PHA, Con A PWM, MLR</td>
<td>not stated MLR: 1 x 10^5 cells from each donor</td>
<td>not stated</td>
<td>ficoll gradient</td>
<td>1 x 10^5 cell/mL</td>
<td>not stated</td>
<td>Not stated 18-24hrs with 3H after 3 day culture</td>
<td>Healthy &amp; myco affected animals</td>
<td>47</td>
<td>Montali <em>et al.</em>, 1998</td>
</tr>
</tbody>
</table>

**Table 5.1: Culture Conditions and Proliferation Responses of Mitogen Stimulated Marsupial Cells.** Data in this table was compiled from a selected range of experiments undertaken across a number of species. ACD acid citrate dextrose anticoagulant; cpm counts per minute; dpm disintegrations per minute; EMEM Eagle’s Modified Essential Medium, FCS Foetal Calf Serum; HI heat inactivated; RPMI Roswell Park Memorial Institute; 3H tritiated thymidine.
5.2 Results
A number of different protocols were assessed for their suitability to isolate, culture and sustain the mitogen stimulation of marsupial lymphocytes and subsequently determine their capacity to mount cell-mediated responses. Once optimised, these conditions were used to promote the production of bioactive molecules by lymphocytes obtained from tammar wallaby peripheral blood, spleen and lymph nodes.

5.2.1 Cell Isolation and Yield
5.2.1.1 Peripheral Blood
Isolation of PBMC by ficoll-paque density gradient separation (2.3.4.2.1a) was the most effective technique used to separate mononuclear cells from granulocytes and erythrocytes. In initial experiments, undiluted blood and blood diluted 1:1 with buffered salt solutions or media were centrifuged over ficoll-paque using the manufacturer’s instructions (500g for 30min at 18°C). Cells recovered using these conditions were routinely contaminated with RBCs and yields were low. After a number of trials, a blood dilution of 1:2 with buffered salt solution and centrifugation conditions of 400g for 20mins at 18°C was successful in recovering buffy coat layers of increased yield and very few contaminating RBCs. Using this technique, between $5 \times 10^6$ and $1 \times 10^8$ PBMC were routinely isolated from 5 to 15mL of tammar wallaby blood, which represents a recovery of approximately 40-60% of the original lymphocyte population number.

Percoll discontinuous gradients were trialled in an attempt to isolate a number of different cell populations within separate layers of the one tube after initial recovery of the leukocyte buffy coat from anticoagulated whole blood. This technique proved useful for removing RBCs and enriching some fractions for monocytes, but was time consuming and required high cell numbers for efficient cell recoveries. Proliferation studies carried out to compare mitogen-driven responses of lymphocytes isolated using ficoll-paque and percoll were not significantly different (data not shown).

When Ficoll-Paque was not available, Histopaque 1.077 was occasionally used as an alternative medium since initial studies found no difference in results obtained using these two solutions. However, cells isolated with Histopaque 1.083, an increased
density medium that was used to improve the yield of monocytes from PBMC, contained up to 10% granulocytes. Therefore, this medium was not used for the routine processing of lymphocytes unless otherwise indicated.

5.2.1.2 Isolation of Lymphocytes from the Lymph Node and Spleen
The recovery of viable cells from spleen tissue was adversely affected by the necessity to break up tissue through a stainless steel sieve. Using this procedure, in two of the three spleen samples processed for isolation of lymphocytes, cell viability was low (30% and 50%). A second ficoll-paque separation was performed to remove dead and dying cells from these preparations and whilst this reduced cell numbers, viability of the remaining lymphocytes was >70%. Between $3.2 \times 10^7$ and $8 \times 10^7$ lymphocytes were recovered from spleen samples in this study.

Lymphocytes isolated from lymph nodes were recovered with higher viability, as less processing was necessary to remove RBCs and connective tissue. $1.5 \times 10^9$ cells at 90% viability were recovered from two enlarged axillary lymph nodes using Method 2.3.4.3.

5.2.1.3 Separation of Lymphocytes using Nylon Wool Columns
Suspension cell preparations (Method 2.3.4.2.3) were passed through nylon wool columns in an effort to separate them into subpopulations with adherent ‘B cell’ properties or non-adherent ‘T cell’ properties (see Method 2.3.4.2.3). With the small numbers of starting cells used for passaging through columns (between $2 \times 10^6$ and $1 \times 10^7$ cells), only a few of these experiments recovered sufficient lymphocytes for use in proliferation experiments (see 5.2.5.3). However, of those experiments performed, $75.7\% \pm 15.8\text{SD}$ of nylon wool recovered cells were non-adherent using this separation procedure (see Table 5.2).
<table>
<thead>
<tr>
<th>Animal</th>
<th>% Non-Adherent</th>
<th>% Adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>592</td>
<td>53.8</td>
<td>46.2</td>
</tr>
<tr>
<td>1612</td>
<td>83.3</td>
<td>16.7</td>
</tr>
<tr>
<td>12F3C0DT</td>
<td>88.4</td>
<td>11.6</td>
</tr>
<tr>
<td>1844</td>
<td>88.8</td>
<td>11.2</td>
</tr>
<tr>
<td>1994</td>
<td>89.7</td>
<td>10.3</td>
</tr>
<tr>
<td>1E722CFT</td>
<td>55.5</td>
<td>44.5</td>
</tr>
<tr>
<td>1657</td>
<td>70.7</td>
<td>29.3</td>
</tr>
</tbody>
</table>

Table 5.2: Recovery of Lymphocyte Populations using Nylon Wool Columns. Tammar wallaby suspension cells were passed through nylon wool columns and separated into non-adherent and adherent fractions. Adherent cells were recovered by mechanical agitation of the nylon wool column. Percentages were calculated as a proportion of total cells recovered.

5.2.2 Cell Culture

Culture conditions for proliferation studies were optimised for parameters such as type of culture media, cell numbers, levels of mitogen, culture volumes and incubation times. Estimation of viable cell numbers and levels of proliferation in these experiments were obtained using the MTT assay (2.3.8.1.1).

5.2.2.1. Media

The ability of different media systems to support unstimulated control cells and stimulated PHA-treated cells is summarised in Table 5.3.

In preliminary experiments, RPMI alone and a cholesterol-containing serum-free media (Hybrimax; Sigma) that contained BSA, transferrin and insulin were used to assess optimum culture conditions. Both of these serum-free systems failed to support control and stimulated cells in culture. Early serum-enriched experiments using RPMI with 10% pooled tammar wallaby serum were abandoned due to the lack of consistent supply. RPMI with 10% low haemaglobin FBS and IMDM with 10% low haemaglobin FBS were compared with a third type of serum-free media.
QBSF®-51, based on IMDM and supplemented with proprietary levels of insulin, transferrin and BSA (Sigma). Serum-free RPMI also generally showed poor survival of unstimulated cells and PHA stimulation in this medium yielded variable results.

Inconsistent results were obtained for the survival of cells in media supplemented with TWS. Whilst media supplementation with some batches of TWS supported control and stimulated cell survival, other batches showed reduced viability of control cells and did not support the proliferation of PBMC after treatment with mitogens when used at the same levels. Both the consistency and supply of tammar wallaby serum was difficult to maintain, so it was discontinued as a media supplement after these initial studies.

In contrast to the variable nature of the responses associated with all other culture media tested, QBSF®-51 media appeared to consistently support both unstimulated control and PHA-stimulated cells. This medium also promoted cell adherence to culture wells, which facilitated the removal of supernatants necessary for effective dissolution of formazan precipitate in the MTT proliferation assay. A number of serum-supplemented systems were successful in terms of sustaining cell viability (Table 5.3) but were incompatible with the MTT solubilisation assay as processing volumes exceeded culture well capacities. In serum-supplemented systems, the majority of lymphocytes were non-adherent at the end of the culture period, so the removal of media was confounded by the presence of the floating MTT precipitate. 1.5mL centrifuge tubes were trialled as an alternative to 96 well plates to allow for an extra centrifugation step to sediment the MTT precipitate (2.3.8.1.1 MTT Suspension Assay). However, this protocol was very time consuming and costly since polystyrene culture tubes were necessary to prevent MTT formazan absorption into the tube material upon addition of the solubilisation buffer. QBSF®-51 was thus the media of choice for use in remaining lymphocyte proliferation experiments.
<table>
<thead>
<tr>
<th>Media</th>
<th>Source of Lymphocytes</th>
<th>Unstimulated Cell Survival*</th>
<th>Stimulated Cell Survival*</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrimax</td>
<td>blood</td>
<td>poor</td>
<td>poor</td>
<td>ND</td>
</tr>
<tr>
<td>RPMI</td>
<td>blood, lymph node, spleen</td>
<td>moderate - poor</td>
<td>low - high</td>
<td>variable</td>
</tr>
<tr>
<td>QBSF®-51</td>
<td>blood, lymph node, spleen</td>
<td>moderate - high</td>
<td>high</td>
<td>&gt; 1</td>
</tr>
<tr>
<td>RPMI +5% FBS</td>
<td>blood</td>
<td>low - moderate</td>
<td>poor</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>RPMI + 10% FBS</td>
<td>blood</td>
<td>moderate - high</td>
<td>moderate - low</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>IMDM + 10% FBS</td>
<td>blood</td>
<td>high</td>
<td>moderate</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>RPMI + 5% TWS</td>
<td>blood</td>
<td>low - moderate</td>
<td>low - moderate</td>
<td>variable</td>
</tr>
<tr>
<td>RPMI + 10% TWS</td>
<td>blood</td>
<td>moderate - high</td>
<td>moderate - high</td>
<td>&gt;1 when serum not inhibitory</td>
</tr>
</tbody>
</table>

*poor < 25%, low 25 – 50%, moderate 51 – 75%, high > 75%. FBS Foetal Bovine Serum; TWS tammar wallaby serum. ND Not Determined.

**Table 5.3: Survival of Tammar Wallaby PBMC in Different Culture Media.** Serum supplemented culture media was inconsistent in the support of both control and stimulated cell survival of tammar wallaby PBMC. In contrast, QBSF®-51 serum free media consistently supported the *in vitro* growth of these cells. ‘Variable’ refers to the total range of responses.
5.2.2.1.1 Morphology of Activated Cells and Colony Formation

Although not all the media supported lymphocyte proliferation over the culture period, lymphocytes were visibly activated by PHA and PWM in all media tested. This was evidenced by a change in shape of resting, spherical cells to a Y-shaped morphology, where both sides of the activated lymphocyte extended blunt pseudopodia (Figure 5.1). In QBSF®-51 serum-free (SF) culture medium, cells transformed to blasts, adopted morphological changes indicative of motile cells, and increased in cell number. Dividing cells were present in cultures after 24 hours contact with PHA and 48-72 hours with PWM when these mitogens were used at both optimal and sub-optimal concentrations.

When lymphocytes were treated with the levels of PHA, Con A and PWM reported in this study, numerous small cell aggregations were formed in the SF medium with the majority of cells associated with a ‘colony’ after 24 hours of culture (Figure 5.3b). The cell clusters formed after PWM treatment were intermediate in number between those formed in serum-free PHA treated systems and those formed in serum-supplemented PHA systems. In contrast to observations from the other two mitogens, non-cluster associated cells were very active when exposed to PWM, as evidenced by changes in cell polarity and cell enlargement.

No evidence of cell aggregations was found in control serum-free cultures (Figure 5.3a), although large cells putatively identified as monocytes and monocyte-derived dendritic cells were apparent in culture within the first 24 hours (see Chapter 4). Fewer, larger cell aggregates were formed in the serum-supplemented systems, most particularly in the Con A treated cultures (Figure 5.3c). However, all cells were not associated with these ‘colonies’ and whilst cells cultured in serum-enriched media displayed active cell morphology after 24-48 hours of culture, evidence of dividing cells was generally not apparent until 72 hours.

Culture in SF media was successful in maintaining the reproduction of cells even though viability decreased with time. At five days in culture, lymphocytes seeded as PBMC increased in mean diameter (± SD) from 9.0 ± 2.6µm (range 5.5 to 17.5µm) to 12.2 ± 2.6µm (range 9.7 to 17.3µm). There were very few smaller lymphocytes present within the culture at this time (Figure 5.2).
Figure 5.1: Tammar Wallaby PBMC in Culture. These micrographs are representative of cell morphology in all experiments where cell activation was apparent. In figure (a), motile cells display a typical Y-shaped morphology (arrow) and elongated cell body. Smaller, ‘resting’ cells are also visible (R). In figure (b), spherical lymphocytes in the early stages of activation (arrowheads) are larger than ‘resting’ cells (R).
Figure 5.2: PBMC were isolated by ficoll-paque density gradient centrifugation and Tammar Wallaby PBMC after Culture in Serum-free Media for 5 Days cultured in QBSF®-51 serum free media. Cell suspensions were placed in 50mL tissue culture plates and incubated at 37°C in humidified air containing 5% CO₂ for five days. After this time, supernatants were removed and cells were fixed in situ with methanol and treated with Diff Quik differential stain. In both figures, lymphocytes are in various stages of activation. Note the large, lightly staining blast cells (B) and degenerating cells such as the apoptotic cell in figure (b) (arrow). Note also the much larger monocyte (M) in figure (a).
5.2.2.1.2 Cell Numbers

Cell numbers between 2.0 x 10^5 and 1.0 x 10^6 cells/well survived in culture and demonstrated a measurable increase when stimulated with PHA. For peripheral blood cell numbers below and above this range, no significant mitogen response was obtained with any of the mitogens tested in this study. Cell counts less than 2.0 x 10^5 cells/well did not survive well in SF medium. Optimal responses for the culture of peripheral blood lymphocytes were achieved using 3.0 x 10^5 to 6.0 x 10^5 cells/well (Appendix One).

5.2.2.1.3 Culture Volume

A culture volume of 200μL was necessary in order to sustain viability of proliferating cells in all experiments. Culture volumes of 150μL were trialled in very early experiments and cells did not survive longer than 24 hours. Culture volumes of 250μL successfully supported both control and stimulated cells but were impractical to use in this assay system due to losses that occurred when microtitre plates were centrifuged according to Method 2.3.8.1.1.

5.2.2.1.4 Incubation Time

QBSF®-51 serum-free media successfully supported the proliferation of PHA stimulated tammar wallaby PBMCs over a 48 hour culture period (Figures 5.9 and 5.10). 24 hour incubation times often resulted in high MTT responses that were judged to be a combination of increases in cellular activity and an increase in cell numbers. Therefore, to avoid misinterpretation of MTT responses, 48 hours was chosen as the standard incubation time. Serum-supplemented systems were variable in their ability to support mitogen-driven responses in this time-frame and were more successful over a 72-96 hour culture period (Figure 5.8).
Figure 5.3: Colony Morphology of Peripheral Blood Mononuclear Cells. These micrographs are representative of colony morphology obtained in both macropod and human cell culture experiments. Figure a) is unstimulated control cells evenly distributed throughout the culture vessel. Figure b) illustrates the pattern of PBMC cell aggregations associated with PHA stimulation in serum-free media. Figure c) shows the typical large discrete cell groups associated with the use of serum-supplemented media. Figures d) and e) illustrate the cell interactions between and within colonies seen in PHA stimulated serum-free cultures (arrows). Micrographs were obtained using inverted phase contrast microscopy. Scale Bar 100µm.
5.2.3 Proliferation Assays

Upon polyclonal or antigenic stimulation, lymphocytes enter the G1 phase of the cell cycle where they enlarge before division of activated lymphocytes occurs in the S (synthesis) phase of the cycle (Alberts et al., 1989). Increases in cell numbers can be quantitated directly by microscopic or mechanical methods, or indirectly by establishing a relationship between total cell numbers and a biological process such as an increase in DNA synthesis or changes in enzyme function (Bybee and Thomas, 1991; Burke et al., 1997).

5.2.3.1 MTT Assay

5.2.3.1.1 Standard Curve

Cell numbers and viability were routinely measured in culture experiments using the MTT assay. A standard curve was constructed that plotted the optical density of dissolved MTT formazan product produced by viable lymphocytes against actual cell numbers counted by trypan blue exclusion.

Initial experiments to determine optimal culture medium properties were performed by direct addition of MTT solubilisation solution (0.1N HCl) to culture wells (Method 2.3.8.1.1 MTT Suspension Assay). This protocol is commonly used in MTT assays and was chosen in order to minimise loss of suspension cells and to reduce assay time. Experiments with RPMI supplemented with tammar wallaby and foetal calf serum were undertaken using this protocol. This assay system proved suitable for cell numbers between 3.5x10^3 and 1.1x10^6 cells/well (Figure 5.4). However, for cell numbers greater than 1.0 x 10^6 cells/well, decreased solubility of the MTT precipitate coupled with changes in cell growth patterns caused a departure from the established linear relationship (Figure 5.4 inset). This protocol therefore proved inappropriate for measurement of cell numbers outside this optimum range.

An attempt was made to overcome this apparent reduced viability at high cell numbers by increasing the volume of medium within the culture wells from 200μL to 250μL to provide extra nutrients to stimulated cells. This increase in culture volume was unsuccessful as the total volume of culture media, together with the required volume of solubilisation solution necessary to dissolve the formazan precipitate, could no longer be contained within the 96-well microplates. The use of culture plates that
could contain larger media volumes, such as 48 well microplates, was considered but was not possible in this study since their use would require an increased number of cells to satisfy minimum density requirements necessary to maintain cell viability. Since insufficient cell numbers were available to allow testing of both control and stimulated cells under these increased volume conditions, this option was not further explored. Therefore, modification of the proliferation assay was undertaken that included complete removal of culture supernatant after the MTT incubation step. This change required a centrifugation step to sediment cells to enable clean removal of the supernatants. A secondary benefit of this change in protocol was that it eliminated sporadic variances in absorbance readings due, most likely, to changes in media volumes caused by losses during incubation.

The change in protocol was assessed using both serum-supplemented and serum-free media. As previously noted, the QBSF®-51 SF medium promoted cell adherence to the culture wells which facilitated removal of supernatants, so this medium was used in conjunction with the change in protocol for the majority of MTT proliferation studies of tammar wallaby lymphocytes.

The relationship between the optical density of the solubilised MTT formazan product and viable cell number in SF medium was found to be linear between $4.0 \times 10^4$ cells/well and $2.2 \times 10^6$ cells/well with a correlation co-efficient of 0.99 (Figure 5.5).
Figure 5.4: MTT Standard Curve for Suspension Assays. 1/10 volume of MTT (5mg/mL) was added to 96 well microtitre plates containing cells between $3.5 \times 10^5$ and $1.1 \times 10^6$ cells/well. Plates were incubated at 37°C for 4 hours in humidified air containing 5% CO₂. 100μL of 0.1N HCl was added to each well and optical density of the dissolved precipitate (formed as a result of mitochondrial processing of MTT) was measured at 550nm (referenced to 655nm). INSET: The assay proved inappropriate for cell numbers greater than $1.0 \times 10^6$ cells/well as solubility of the precipitate and kinetics of cell growth in this media system caused a departure from the established linear relationship (arrow). Original cell assay included cell numbers up to $1.5 \times 10^6$ cells/well.
Figure 5.5: MTT Standard Curve. 1/10 volume of MTT (5mg/mL) was added to 96 well microtitre plates containing cells between $4.0 \times 10^4$ and $2.2 \times 10^6$ cells/well. Plates were incubated at 37°C for 4 hours in humidified air containing 5% CO$_2$ and then centrifuged to remove supernatant. Optical density of the dissolved precipitate (formed as a result of mitochondrial processing of MTT) was measured at 550nm (referenced to 655nm).
5.2.3.1.2 Validation of MTT Assay

To validate the suitability of the MTT assay for measurement of marsupial lymphocyte proliferation, PHA at levels of 25µg/mL and 50µg/mL were applied to tammar wallaby PBMC in SF medium. These levels were selected since they are used routinely in marsupial lymphocyte studies (Table 5.1) and allowed direct comparison of lymphocyte proliferation values using both the MTT and [³H]-thymidine assays.

Figure 5.6 illustrates the proliferation results obtained from the lymphocytes of eight different animals after stimulation with PHA at 25µg/mL. Optical density of both control and stimulated cells are shown here in order to demonstrate the low standard deviation associated with this assay (less than 10% of mean OD for all analyses) and the obvious differences between baseline and PHA-treated cultures ($P<0.05$ for all animals). Cells from animals shown in this figure recorded stimulation indices between 1.26 (animal 8021) and 2.21 (animal 1652). Included in this data are three SIs from animal 8021 to illustrate the within-animal variation between assay times. Whilst the difference in control values for animal 8021 was significant between sampling dates ($P < 0.010$), the difference in stimulation indices between control and PHA-treated cells at these times was not significantly different ($P = 0.160$). Some of the data in this figure appears in the time course and dose-response experiments presented in later sections.

5.2.3.2. Tritiated [³H]-Thymidine Incorporation Assay

The [³H]-Thymidine Assay was used in this study to generate data using a method previously employed for measurement of marsupial proliferation for comparison with results obtained using the MTT assay. Raw counts obtained using tritiated thymidine incorporation, adjusted for media and scintillant controls, ranged from 48cpm (animal 1657) to 1 949cpm (animal 1378a) for unstimulated cells and from 234cpm (animal 1E72) to 12 738cpm (animal 1378a) for PHA-stimulated cells.

In order to directly compare data derived from both the radiolabelled and colourimetric assays, results from [³H]-thymidine experiments were log transformed. This step was necessary since the incorporation of radiolabelled thymidine into DNA occurs exponentially (Kristensen et al., 1982b) whilst mitochondrial processing of
MTT is a linear process (Mosmann, 1983 and this study Figure 5.5). Stimulation indices obtained using both the MTT and \([^3H]\)-thymidine assays performed on identical samples are recorded in Table 5.4. When cultured at approximately 5 x 10^5 cells/well, both 25μg/mL and 50μg/mL PHA treatments increased baseline \([^3H]\)-thymidine incorporation levels compared with control wells. Of the eleven peripheral blood lymphocyte stimulation results obtained using the \([^3H]\)-thymidine assay, six samples possessed SIs greater than 3.0. In all samples assessed using both proliferation methods, SI results obtained from the MTT assay were greater than 1.20 where SI values for \([^3H]\)-thymidine cultures were greater than 3.0. This relationship is illustrated graphically in Figure 5.7 where the correlation between ranked values obtained on the same samples using the different assays is significant, with an R^2 value of 0.61.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cell Number (cells/well)</th>
<th>([^3H])-thymidine</th>
<th>MTT</th>
<th>([^3H])-thymidine</th>
<th>MTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1612</td>
<td>5.4 x 10^5</td>
<td>NT</td>
<td>NT</td>
<td>6.8</td>
<td>1.20</td>
</tr>
<tr>
<td>1657</td>
<td>5.1 x 10^5</td>
<td>13.3</td>
<td>1.40</td>
<td>12.0</td>
<td>1.31</td>
</tr>
<tr>
<td>12F3 ▲</td>
<td>5.8 x 10^5</td>
<td>3.9</td>
<td>1.21</td>
<td>2.4</td>
<td>1.07</td>
</tr>
<tr>
<td>1E72</td>
<td>5.0 x 10^5</td>
<td>2.4</td>
<td>1.03</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1844</td>
<td>5.0 x 10^5</td>
<td>10.0</td>
<td>1.66</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1378a ▲</td>
<td>8.0 x 10^5</td>
<td>6.5</td>
<td>1.30</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1378b</td>
<td>6.4 x 10^5</td>
<td>2.7</td>
<td>0.84</td>
<td>1.9</td>
<td>0.68</td>
</tr>
</tbody>
</table>

NT: Not tested ▲: High control values for tritiated thymidine incorporation

Table 5.4: Comparison of Stimulation Indices (SI) of PHA-treated PBMCs using the MTT and \([^3H]\)-Thymidine Proliferation Assays. SIs were calculated as the value of PHA-treated cultures divided by the value of control cultures measured as cpm for tritiated \([^3H]\) thymidine incorporation or absorbance units (AU) for MTT bioreduction. Results were calculated from the mean of triplicate samples for both test and control samples.
Figure 5.6: Optical Density of Control and PHA-treated Lymphocytes using the MTT Assay. Cells were cultured in serum-free media at $4.5 \pm 1.5 \times 10^5$ cells/well both with and without PHA at 25$\mu$g/mL and incubated for 48 hours in humidified air containing 5% CO$_2$. The OD of dissolved formazan precipitate, formed after a four hour incubation with 1/10 volume of MTT, was measured at 550nm referenced to 655nm.

Figure 5.7: Relationship Between the [$^3$H]-thymidine and MTT Incorporation Assays. The relationship between ranked values obtained on the same samples using different assays is expressed here in the linear regression line as $S_i^{MTT} = 0.9318 \times S_i^{Tth} - 0.1077$ with a significant correlation between the two proliferation values ($R^2 = 0.61$).
5.2.4 Responses to Mitogens
Tammar wallaby PBMC were assessed for their ability to proliferate when treated with PHA, Con A and PWM. A small number of experiments were also performed to investigate the response to the phorbol ester, PMA, the calcium ionophore, Ionomycin and the B cell mitogen, LPS.

5.2.4.1 Phytohaemagglutinin (PHA)
PHA-M, one of a number of different grades of this mitogen, was used in this study since earlier marsupial mitogen experiments were performed using this particular grade (Prasad et al., 1971). With the exception of a small number of apparently isolated instances, all concentrations of PHA used in this study caused measurable cell proliferation. In some assays, a concentration of this mitogen at 100μg/mL was occasionally cytotoxic (data not shown). The concentration of mitogen that caused the maximal proliferation response varied between and within animal samples for PHA concentrations of 5, 10, 25 and 50μg/mL over the period of study. However, PHA at levels of 25μg/mL caused the most consistent and reproducible results (see Figures 5.6 and 5.9).

In PBMC culture experiments using RPMI supplemented with either 5% or 10% TWS or FCS, optimal proliferative effects of PHA were achieved between 72 and 96 hours. At 96 hours, many of the control cell population were not viable, and whilst the ratio of stimulated to unstimulated cells remained high, the low control values artificially inflated the proliferation response when compared with results obtained using shorter incubation times. In contrast, control cell survival in serum-free medium at 48 hours for all culture experiments (including those outside the range of optimal cell numbers), expressed as a function of original cell number, was 68% ± 23% (mean ± SD; n = 14). After 48 hours in the PHA stimulated cultures, this number rose to 125% ± 51% (mean ± SD; n = 14) when compared with control cell numbers at time zero. This doubling of cell numbers proved the suitability of this culture system for proliferation studies and was consistent for this mitogen.

Time course studies for both serum-supplemented and serum-free media were performed using PHA as the test mitogen. The results from the culture of tammar
wallaby 6693 PBMC cultured in RPMI with 5% pooled tammar serum for 48, 72, 96 and 120 hours after treatment with PHA at between 2.5 and 20.0μg/mL are shown in Figure 5.8. When cultured in this media system, tammar wallaby PBMCs showed optimum proliferation when incubated for 96 hours and stimulated with PHA concentrations of up to 20μg/mL. These early experiments were performed with low levels of mitogen to verify literature reports that marsupial cells required higher levels of PHA than eutherian mammals for in vitro proliferation. In this figure, the highest level of PHA (20μg/mL) was clearly the most stimulatory and in other experiments, where proliferation in media-supplemented systems was successful, levels of PHA at 25μg/mL and 50μg/mL were routinely employed.

A set of time-course experiments was also undertaken with cells cultured in serum-free media. For most samples, a full time-course could not be carried out due to limited cell numbers, though 48 and 72 hour cultures were routinely performed. Figure 5.10 represents the PHA proliferation response of PBMC from animal 12F3C0DT when cells were cultured in SF media for 1 to 5 days. It is clear from this figure that MTT responses are maximal for the 24 hour incubation time. However, when early activation effects were discounted and relative numbers of dividing cells were confirmed by visual inspection, the 48 hour culture period supported the highest MTT proliferation responses.
Figure 5.8: MTT Suspension Cell Proliferation Assay in RPMI + 5% Pooled Tammar Serum. Early assays assessed pooled tammar wallaby serum for suitability as a culture additive in serum-supplemented media systems. Control and PHA-treated cells were incubated at 37°C in humidified air containing 5% CO₂ for 2 to 5 days before proliferation responses were measured using the MTT suspension assay. In this media system, proliferation was optimal at 72 to 96 hours.
Figure 5.9: Proliferation of Tammar Wallaby PBMC in response to PHA. Cells from three separate animals were cultured in triplicate for 48 hours in serum-free media at 4.0 x 10^5 cells/well and stimulated with 10, 25, 50, 75 and 100μg/mL of PHA. Cultures were incubated at 37°C in humidified air containing 5%CO₂ and proliferation responses were measured using the MTT assay.

Figure 5.10: Time Course for Proliferation of Tammar Wallaby PBMC in response to PHA. Cells from animal 12F3CODT were cultured in serum-free media for 1 to 5 days under standard conditions. Limited cell recovery from restricted sample volumes rarely allowed a complete time course.
5.2.4.2 Concanavalin A (Con A)

Proliferation assays using Con A were performed only when cell numbers were available in excess of requirements for other analyses (e.g. standard curve construction, media comparison, PHA stimulation). As a consequence, there were very few opportunities to test the same animal on more than one occasion using more than one concentration of this mitogen. Cell numbers were limited to levels pre-determined for other tests so proliferation results are expressed here as percentage of surviving control cells. This method of presenting data is also more appropriate than OD values in this instance, since both the suspension MTT assay and standard MTT assay were used to generate Con A proliferation data.

Unlike PHA, Con A was not consistently mitogenic in the serum-free medium QBSF®-51. The variation in proliferation results was considerably higher in Con A stimulated cells in both serum and serum-free systems when compared with those achieved using PHA. Of the 55 separate tests carried out, 18 yielded a standard deviation of greater than 10%.

Lymphocyte proliferation using this mitogen was best supported in serum-supplemented media composed of RPMI with 10% low haemaglobin FBS. Most PBMC Con A proliferation experiments were undertaken using 10, 20 or 25μg/mL since these concentrations have routinely been used by other marsupial researchers (Ashman et al., 1976; Brozek et al., 1992; Buddle et al., 1992). Proliferation results obtained from two different animals in different media are shown in Figure 5.11 to demonstrate the range of proliferation values obtained in these systems. In this figure, data was chosen from experiments where control cell survival was optimal so that proliferation values were not artificially inflated.
Figure 5.11: Proliferation of Tammar wallaby PBMC in response to Concanavalin A. Cells were cultured in RPMI +10% FBS and QBSF®-51 serum-free media after treatment with mitogen and incubated for 48 hours at 37°C in humidified air containing 5% CO₂. 1/10 volume of MTT was added to each well and the plates were incubated for a further four hours. The OD of the dissolved formazan precipitate formed during this time was measured and proliferation responses were expressed as the %Con A treated cells compared with control cells incubated under the same conditions. Proliferation responses were routinely higher in serum-enriched media in response to this mitogen.
5.2.4.3 Pokeweed Mitogen (PWM)

PWM was used to successfully stimulate PBMC in both serum-supplemented and serum-free media. Levels of this mitogen necessary for cell proliferation were lower than those required for PHA proliferation using the same culture conditions (Figures 5.12 and 5.13).

Although a typical dose-response curve was not obtained, optimum proliferation of tammar wallaby PBMC for PWM-driven responses occurred in SF media for mitogen doses between 1.0 and 5.0μg/mL when cells were cultured for 48 to 72 hours (Figure 5.14). However, a wide range of PWM concentrations were stimulatory for tammar PBMC and lower levels of this mitogen, down to 50ng/mL, caused significant proliferation (SIs between 1.77 and 1.88) in some samples. Dividing cells were clearly visible between 48 and 72 hours of culture, which confirmed the optimal proliferation time for culture with this mitogen.
Figure 5.12: PWM-Driven Proliferation of Tammar Wallaby PBMC in RPMI + 10% FBS. Dose response curves for four different animals are shown here for 5 x 10^5 cells/well cultured for 72 hours at 37°C in humidified air containing 5% CO₂. All treatments between 1μg/mL and 5μg/mL were carried out in triplicate and proliferation was determined using the MTT assay.

Figure 5.13: PWM-Driven Proliferation of Tammar Wallaby PBMC in Serum-free Media. MTT assays were performed on 5 x 10^5 cells treated with PWM concentrations of 2.5, 5, 10 and 20μg/mL cultured for 48 hours at 37°C in humidified air containing 5% CO₂. Results are shown for three animals and are derived from triplicate samples.
Figure 5.14: Time Course for PWM-Driven Proliferation of Tammar Wallaby PBMC. 4.8 × 10^5 PBMC/well from tammar wallaby 12F3CDT were treated with PWM at 2, 5, 10 and 25 μg/mL in serum-free media and incubated under standard conditions in 96 well flat-bottomed microtitre plates for 1-5 days. The optical density of treated and untreated cells was obtained using the MTT assay. Cells were not treated with 25 μg/mL on day 5 due to insufficient sample.

<table>
<thead>
<tr>
<th>PMA(ng/mL)</th>
<th>% of Control mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>100ng/mL</td>
<td>262 ± 5</td>
</tr>
<tr>
<td>150ng/mL</td>
<td>263 ± 14</td>
</tr>
<tr>
<td>200ng/mL</td>
<td>273 ± 6</td>
</tr>
<tr>
<td>Suspension Cells</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>25ng/mL</td>
<td>167 ± 1</td>
</tr>
<tr>
<td>50ng/mL</td>
<td>170 ± 6</td>
</tr>
<tr>
<td>75ng/mL</td>
<td>162 ± 2</td>
</tr>
<tr>
<td>100ng/mL</td>
<td>166 ± 3</td>
</tr>
</tbody>
</table>

Table 5.5: Proliferation Responses of Tammar Wallaby PBMC and Suspension Cells to PMA. 4.8 × 10^5 PBMC and monocyte-depleted PBMC preparations (suspensions cells) from animal 1994 were incubated under standard conditions for 48 hours in serum-free medium and stimulated with various levels of PMA. Proliferation responses were obtained from triplicate wells and measured using the MTT assay. Results are expressed as the mean percentage of control cells where the OD of stimulated cells was compared with the OD of control cells for the same sample.
5.2.4.4 PMA and Ionomycin

Phorbol myristate acetate was used in isolation and in conjunction with the ionophore, ionomycin, to investigate lymphocyte proliferation responses. Dose-response curves were not established for these mitogens in combination due to cell number constraints. However, when tested separately, strong proliferation responses were obtained for PMA applications between 25 and 200ng/mL (see Table 5.5) and for ionomycin at 50, 75 and 100ng/mL (% of control values, mean ± SD: 126 ± 7, 155 ± 7 and 150 ± 3 respectively). When tested at 20ng/mL across three animals, PMA caused an increase in cell number of 3.2% ± 2.8%SD which was not significant at P<0.05. Ionomycin was stimulatory at dosages of 50ng/mL when tested on the cells of three animals on four separate occasions (130 ± 19, 150 ± 9 and 198 ± 9% of controls) for 1657, 1994 and 8021 respectively. The proliferation of cells from animal 1994 when treated with ionomycin at 25ng/mL was not significant because of a large range in MTT values for this sample (91% ± 14% of control cells).

Other researchers have demonstrated synergistic responses with the PMA levels used in the current study in combination with ionomycin at levels of 1 and 5μg/mL (Rose et al., 1992). When tested in isolation or in combination with optimum PMA levels, ionomycin concentrations of 1 and 5μg/mL proved toxic to tammar wallaby lymphocytes, with between 11 and 21% of stimulated cells surviving compared with control cells. Since ionomycin and PMA independently stimulated M. eugenii lymphocytes, no further combinatorial studies were performed for these mitogens.

5.2.4.5 Lipopolysaccharide (LPS)

Lipopolysaccharide is a B cell mitogen and since humoral responses were not the focus of this study, LPS-driven lymphocyte responses were not extensively investigated. However, where cell numbers permitted, a small number of experiments were undertaken to assess the suitability of serum-free culture conditions on PBMC treated with various levels of LPS. Concentrations of LPS greater than 50μg/mL caused measurable increases in OD over a 48 hour culture time in two separate experiments for animal 1994 (Figure 5.15). A set of comparative media assays were also undertaken to investigate longer culture times since B cells are generally cultured in vitro for more than five days (Rose et al., 1992). Concentrations of 10μg/mL, 50μg/mL and 100μg/mL of LPS were used in both serum-supplemented (RPMI +10%
FBS) and serum-free media and incubated for 6 days. Cultures contained $1 \times 10^6$
PBMC/well in order to increase the number of B cells delivered in the PBMC cell
suspension. The viability of cells cultured in both serum-free and 10% FBS
supplemented RPMI media was maintained compared with control cells when treated
with LPS at doses greater than 50µg/mL. However, whilst there was a trend for
increasing proliferation as treatment concentrations increased, this increase was not
significant and relatively large SDs were apparent (Figure 5.15). Visual inspection of
serum-free cultures showed monocyte deterioration during this incubation time. This
was clearly visible as membrane blebbing in large mononuclear cells of the treated
cultures (see Figure 4.7). LPS was therefore considered toxic for monocytes in this
culture system.
Figure 5.15: Proliferation Response of Tammar Wallaby PBMC to Lipopolysaccharide. Cells from animal 1994 were cultured in serum-free media at 4.8 x 10^4 cells/well and incubated at 37°C for 48 hours in humidified air containing 5% CO₂. 1/10 volume of MTT was added to each well and the OD of dissolved formazan product was measured after removal of the culture supernatant. Results were obtained from triplicate samples from each of two separate experiments.
5.2.5 Whole Blood Assays

In addition to investigations of the proliferative capacity of isolated PBMC, this study also investigated proliferation responses using whole blood samples. This work was undertaken to establish a method suitable for use in studies where only small blood volumes are available e.g. for younger, smaller animals and for rare samples from endangered species. Using a modified MTT assay (Method 2.3.8.1.1, Whole Blood Assay), anticoagulated whole blood volumes between 2μL and 100μL were tested for proliferation responses using mitogen levels as described in Method 2.3.8.2.2. Results obtained from blood volumes below 10μL were not reproducible. Blood volumes of 20μL or greater were problematical because of interference due to larger numbers of RBCs. Proliferation results obtained using blood volumes of 10μL proved optimal for this assay and these results are summarised in Table 5.6.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood Volume (μL)</th>
<th>Mean Stimulation Index (MTT assay)</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (50μg/mL)</td>
<td>10</td>
<td>0.96</td>
<td>0.27</td>
<td>8</td>
</tr>
<tr>
<td>Con A (10μg/mL)</td>
<td>10</td>
<td>1.51</td>
<td>0.35</td>
<td>4</td>
</tr>
<tr>
<td>Con A (25μg/mL)</td>
<td>10</td>
<td>1.13</td>
<td>0.17</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5.6: Proliferation Responses of Whole Blood Cultures from the Tammar Wallaby. Triplicate samples of a 1/10 dilution of anticoagulated whole blood were used to assess the whole blood proliferation responses of *M. eugenii* leukocytes to PHA and Con A. Proliferative activity was measured using the MTT assay. Note the depressed PHA response. *n* = number of animals tested.

5.2.6 Comparative Assays

A small number of assays were performed on human and sheep blood samples to allow validation of the culture techniques and to generate data for comparison with marsupial lymphocytes cultured under similar conditions.
5.2.6.1 Human

5.2.6.1.1 Whole Blood Assays

A tritiated thymidine proliferation assay was performed on tammar and human whole blood samples to allow comparison of this technique with previous data and the whole blood MTT assay developed in this study. 5 and 10μL volumes of whole blood from both human and tammar (8021) subjects were diluted in RPMI (without phenol red) and incubated with and without mitogens for 48 hours. Very large standard deviations resulted from assays using 5μL blood volumes, so they are not reported here. In cultures using 10μL of blood, tammar wallaby control cells demonstrated higher thymidine incorporation than human cells, which may reflect the absolute numbers of lymphocytes present in the samples (8.2x10^6 cells/mL in 8021 and 3.2x10^6 cells/mL for human blood). This difference was not maintained after stimulation with mitogens and although tammar wallaby leukocytes showed a proliferation response with PHA, both PHA and Con A responses were markedly lower than for human whole blood samples under the conditions of this test (Table 5.7).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tammar (cpm ± SD)</th>
<th>Human (cpm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>530 ± 301</td>
<td>297 ± 79</td>
</tr>
<tr>
<td>PHA (25μg/mL)</td>
<td>1812 ± 659</td>
<td>14952 ± 523</td>
</tr>
<tr>
<td>Con A (5μg/mL)</td>
<td>431 ± 113</td>
<td>4564 ± 1121</td>
</tr>
</tbody>
</table>

Table 5.7: Tritiated Thymidine Incorporation of Mitogen-treated Human and Tammar Wallaby Whole Blood Cultures. 10μL of anticoagulated whole blood was diluted in RPMI and treated with and without mitogens for 48 hours. Results are the averages of triplicate samples.

5.2.6.1.2 PBMC Assays

Human PBMCs were isolated by ficoll-paque density gradient centrifugation (2.3.4.2.1) and cultured at 3.9 x 10^5 cells/well in both serum-free and RPMI 1640 + 10% FBS media. Visual inspection of both culture systems revealed that cell colony aggregations were similar to those formed by tammar wallaby cells (Figure 5.3).
mitogen-driven proliferation trends in the different media were also similar to tammar wallaby experiments, where PHA responses were optimal in QBSF®-51 serum-free media whilst Con A responses were maximal in serum-supplemented media (Figure 5.16).

5.2.6.2 Sheep
Lymphocytes were also isolated from a number of sheep blood samples kindly donated by Dr. Bruce Brown (CSIRO Animal Division, Prospect, Australia). After separation of the leukocyte layer from whole blood (Method 2.3.4.2.1c), lymphocytes were recovered using ficoll-paque density gradient centrifugation (Method 2.3.4.2.1a). Sheep cells were cultured in triplicate in both serum-supplemented media and serum-free media for 72 hours. In one set of experiments using RPMI + 5% FBS, SIs of 1.67 and 1.60 were obtained for 5 x 10^5 lymphocytes/well when treated with PHA at 20μg/mL and Con A at 5μg/mL respectively. Cells isolated from a different animal, cultured in SF medium at 8 x 10^4 lymphocytes /well were not viable after this same time period. Experiments undertaken on a different set of sheep samples were unsuccessful in RPMI +10% FBS at cell numbers of 1.5 x 10^4 cells/well but yielded a SI of 1.65 for cells cultured in SF medium at 1.4 x 10^4 cells/well. The low viability of sheep cells in long periods of culture for SF medium and at low cell numbers in serum-supplemented systems was consistent with the findings in marsupial culture experiments.
Figure 5.16: Proliferation of Human PBMC Cultured with both Serum-Free and Serum-supplemented Media. $3.9 \times 10^4$ cells/well were cultured in both serum supplemented and serum free media. Mitogens were added at the levels indicated and cultures were incubated at $37^\circ C$ in humidified air containing 5% CO$_2$ for 48 hours. Proliferation responses were measured in triplicate wells for both control and mitogen-treated samples using the MTT assay. Note the optimum Con A response in serum-supplemented media.
5.2.6.3 Proliferation of Cells separated using Nylon Wool

In an attempt to identify the functional capacity of cells separated using the nylon wool technique, mitogen proliferation assays were undertaken on both non-adherent and adherent cell fractions. Figure 5.17 shows the relative responses of the non-adherent lymphocytes from five animals in response to PHA in serum-free media, after fractionation of their lymphocytes into putative T-enriched and B-enriched pools.

When non-adherent cells were cultured with PHA, strong agglutination of cells was evident after approximately 4 hours of culture, including those where SIs were less than one. For all adherent cells, a number of small cell aggregations formed when treated with this mitogen but, with the exception of cells from animal 592, these cells showed little mitogen proliferative response. Both adherent and non-adherent cells from animal 592 appeared to proliferate strongly in response to PHA. However, the adherent cell response was most likely due to inefficient separation of the two fractions, since strong agglutination of cells was apparent in these wells.

5.2.6.4 Proliferation of Lymphocytes isolated from Tammar Wallaby Lymph Nodes

Lymphocytes were isolated from tammar wallaby lymph nodes according to method 2.3.4.3. Isolated cells were then stimulated with a range of mitogen concentrations that included the type and level of mitogen that yielded optimum responses for *M. eugeni* peripheral blood cells, as well as those used in previously published studies of lymphocytes isolated from the lymph nodes of other marsupials.

To assess the most suitable media for culture, cells from animal 1538 were incubated in both serum-free (1.8 x 10^6 cells/well) and 10% FBS-supplemented RPMI media (2.1 x 10^6 cells/well) and treated with PHA at 25 and 50μg/mL and Con A at 10 and 25μg/mL. Figures 5.18 and 5.19 demonstrate the relationship between mitogen treatments and the relative proliferation responses of lymph node cells when cultured for 48 hours in both media. In these experiments, PHA-driven proliferation was optimal in serum-free media and Con A promoted proliferation was optimal in media-supplemented RPMI. PWM responses were also shown to be stimulatory in SF culture at 5μg/mL (Figure 5.19).
Cells from animal 1958 were processed to remove monocytes by overnight adherence and subsequently tested for proliferation response to PHA and PWM. Proliferation values for PHA were similar after removal of adherent cells, whilst the PWM response decreased from 150% to 120% of control values ($P<0.01$). Consistent with mitogen-driven proliferation culture responses of peripheral blood cells, higher baseline responses of control cells in serum-free media were apparent and larger standard deviations were associated with Con A treatments compared with those of PHA and PWM (Figures 5.18 and 5.19).
Figure 5.17: Proliferation Response of Nylon Wool Separated Cells to PHA in Serum-free Medium. Non-adherent cells were treated with PHA at 25µg/mL except for animal 592, which was treated at 50µg/mL. Although decreases in cell survival compared with control cells were apparent in 1994, 12F and 1657, cells within these fractions formed cell aggregates consistent with proliferative activity in other culture wells.
Figure 5.18: Proliferation of Lymph Node Cells to Mitogens in Serum-
Supplemented and Serum-Free Media. Results were obtained using the standard MTT
assay for both media systems and are presented as the mean ± SD of triplicate cultures.

Figure 5.19: Proliferation of Lymph Node Cells to Mitogens in Serum-Free
Media. Results are presented as the mean % of control values obtained using the MTT assay ± SD of triplicate cultures. Insufficient cells were available for PWM testing of sample 1538.
5.2.6.5 Proliferation of Cells isolated from the Spleen

Lymphocytes isolated from tammar wallaby spleen preparations were cultured for both 24 and 48 hours to assess optimal culture conditions in serum-free media. Consistent with results from peripheral blood cells, MTT responses were higher in 24 hour cultures (see Figure 5.20) but dividing cells were more abundant in 48 hour tests, so this was chosen as the routine culture time. The relative mitogen responses were most notable in Con A stimulated cells that only contained low numbers of dividing cells after 48 hours, illustrated by the low response to Con A after this time (Figure 5.20).

Despite the limited availability of samples, a number of different mitogens and mitogen concentrations were assessed for their proliferative capacity (Figure 5.21). Spleen lymphocytes from the clinically healthy animals 1958 and 1935 were stimulated by PHA but this response was depressed in cells isolated from animal 209 which was euthanased due to ill-health. Con A levels of 10µg/mL were stimulatory for cells from animal 209 but when applied at 25µg/mL on cells from animal 1935, this mitogen was cytotoxic. The Con A/PHA response in SF media for animal 209 was reversed compared with responses from normal peripheral blood and spleen samples cultured in this media.

In summary, similar to PBMC, the optimum cell number for culture and proliferation of spleen lymphocytes was found to be $4.5 \pm 1.5 \times 10^5$ cells/well. For lymph node cells, higher numbers of cells were required in order to sustain proliferation (see Appendix Two).
Figure 5.20: Proliferation of Mitogen-Stimulated Spleen Cells for 24 and 48 hours. Cells from animal 1935 were treated with PHA and Con A in serum-free media for 24 and 48 hours and processed using the MTT assay. Note the depressed Con A response at 48 hours.

Figure 5.21: Proliferation of Mitogen-Stimulated Tammar Wallaby Spleen Cells. Lymphocytes from the spleens of three animals were treated with various mitogens for 48 hours in serum-free media. Note the large SD and the lower SI of PHA treated cells and the high SI of Con A stimulated cells from animal 209. Data from animal 1935 shown in Figure 5.12 is included here for comparison. All mitogen concentrations are in µg/mL.
5.2.7 Lymphocyte Responses to Cell Culture Supernatants

The polyclonal activation of lymphocytes due to mitogen treatment in vitro leads to the expression of many different cytokine genes (Kelso, 1998). During the process of activation, cells move from the resting stage of the cell cycle (G0) through to a period of DNA synthesis (S). This passage requires the progression through a restriction point (R) after which cells no longer require an exogenous activation stimulus (Kaplan et al., 1997). Thus, cytokines produced after in vitro polyclonal activation may be collected in the supernatants of lymphocyte cell cultures if lectin-containing media is removed and replaced with fresh, mitogen-free media. In eutherian culture systems, detectable cytokines are secreted into cell culture supernatants approximately 5 hours after stimulation (Kaplan et al., 1997) whilst in avian systems, the maximal activity of IL-2, and therefore the implied maximal secretion of this lymphokine, occurs at 24 hours (Bertram et al., 1997).

Levels of mitogen that proved to be stimulatory in tammar wallaby lymphocyte proliferation studies were used to stimulate the secretion of bioactive components into the culture supernatants of mitogen-treated marsupial cells. To this end, lymph node, spleen and PBMC mononuclear cell cultures were treated with PHA at 25μg/mL, Con A at 10μg/mL and PWM at 2μg/mL. PHA stimulation experiments were performed in QBSF®-51 serum-free conditions and Con A-driven proliferation was performed in serum-supplemented RPMI.

After 24 hours in culture, supernatants containing putative bioactive factors were harvested from treated cells along with control cell supernatants incubated under the same conditions. Cells were washed three times with PBS to remove traces of residual treatments and media was added back to mitogen-activated cells and then collected after a further 24-48 hours incubation. Cell culture supernatants were subsequently fractionated to enrich for compounds present in the molecular weight range of most cytokines (<50kDa) according to method 2.3.8.3.

In all supernatant culture proliferation experiments, control wells treated with mitogen (PHA at 25μg/mL or PWM at 2μg/mL) were included to verify the proliferative capacity of test cells. In these assays, supernatant activity was defined as bioactive (stimulatory or inhibitory) if the OD resulting from test fractions was greater or less
than 1SD above or below the mean of that measured in controls tested at the same
time. In preliminary studies, unfractionated supernatants harvested from PHA, Con A
and PWM treated lymph nodes, spleen and PBMCs were used at various dilutions to
screen for functional characteristics that confirmed the presence of bioactive
properties.

The majority of the supernatants from mitogen-stimulated lymphoid cell cultures
showed significant bioactivity ($P < 0.05$) when used at dilutions between 1/5 and 1/50,
although the most effective dilution was not consistent across all samples (data not
shown). In a preliminary screening using 1/5 and 1/10 dilutions of control and
stimulated supernatants recovered after Con A stimulation, using QBSF®-51 as the
base medium, 1/5 and 1/10 dilutions of Con A-treated supernatants and the 1/5 test
control showed significant proliferation when compared with control PBMCs. The
1/10 test control dilution was not significantly different from fresh control cells
($P = 0.126$) although the mean OD value for this test was still higher than the control
(0.324±0.014 versus 0.299±0.019). Whilst these results suggest that supernatants
contained stimulatory factors, the functional activity of Con A supernatants was not
explored further as the effects of undefined growth factors present in the FBS used to
supplement the original growth medium could not be separated from the effects of the
putative stimulatory factors present in the recovered supernatants.

Bioactive-positive supernatants originally prepared in serum-free medium were
subsequently separated using spin columns into fractions enriched for molecules
<10kDa and those that fell between 10kDa and 30kDa or 10kDa and 50kDa. As a
quality control step, Polyacrylamide Gel Electrophoresis (PAGE) was undertaken on
separated fractions to confirm that high molecular weight molecules were removed
from test preparations. When stained with coomassie blue and silver protein stains
(2.3.8.5.2), PAGE demonstrated that there were no detectable large molecular weight
molecules present in the supernatants post fractionation (not shown).

After refinement of culture conditions and molecular weight fractionation of
supernatants, testing of bioactive factors in the supernatants of cultured lymphocytes
was undertaken in basal RPMI media. This modification was introduced since this
media did not support the proliferation of tammar wallaby lymphocytes without
supplementation (Table 5.2) and would therefore be more sensitive in the detection of bioactive factors in any treatments tested. Supernatant fractions were tested at dilutions of 1/10, 1/20 and 1/50 in this basal medium. Control PBMC cells were treated with the same volume of frozen, thawed, size-fractionated serum-free media that was present in the original harvested supernatants, in order to supply trace bioactive factors that may have been present in the serum-free media rather than the growth factors contained within test fractions. Supernatants with high levels of stimulatory activity were associated with cell aggregations or small colonies forming within the culture vessel. Increased activity of treated cells was clearly evident with cells adopting active cell morphology (Figure 5.22) similar to mitogen-treated samples (see Figure 5.1).

Both stimulatory and inhibitory factors were found to be present in the fractions tested at 1/10 dilutions in basal RPMI (Figure 5.23). When tested on freshly isolated PBMC of three different animals, anti-proliferative factors were present in both the test control samples and the <10kDa PHA treated fractions harvested from lymph node cultures from animal 1958 (P≤0.001). Fractions displaying proliferative activity were derived from control and PHA-treated supernatants pooled from all lymphoid cell cultures to maximise the use of materials. The control fractions >10kDa<50kDa and <10kDa as well as the PHA fractions >10kDa<30kDa and PHA<10kDa showed significant stimulatory effects on freshly isolated PBMC (P<0.05). There were, however, three tests that were not significant when used at these dilutions. Whilst further testing of PHA>10kDa pooled fractions was not possible due to depletion of this test material, the control <10kDa sample from animal 12F3 was significantly proliferative (P=0.038) and the PHA <10kDa pooled sample was stimulatory at P=0.09 when both were tested at a dilution of 1/20.
Figure 5.22: Inverted Phase Micrograph of Tammar Wallaby Suspension Cells after Treatment with Mitogen-Stimulated Cell Supernatant. 1/10 dilution of pooled >10K<30K supernatant was added to freshly isolated PBMCs from animal 1400 and cells were incubated at 37°C in humidified air containing 5% CO₂ for 24 hours. In this micrograph, ‘resting’ lymphocytes are spherical. Note the elongated cell forms and cytoplasmic projections characteristic of active lymphocytes (arrows).
Figure 5.23: Proliferation Response of Tammar Wallaby PBMC to Supernatants Harvested from Cultured Mononuclear Cells. Three separate experiments on different animals were carried out to determine the proliferative effect of supernatants collected from both control and mitogen stimulated mononuclear cells cultured in serum-free media. In this figure, all control cells were treated with a 1/10 dilution of the test agent. Treatments shown here include supernatants harvested from control cells at the time of original culture (testcontrol) and supernatants collected from PHA (25µg/mL) stimulated cells. Supernatants from 1958 lymph node cells and pooled PBMC, spleen and residual lymph node cultures were size separated to isolate fractions enriched for molecules < 10kD and between 10kD and 30 or 50kD. See text for details. Proliferation responses were measured in triplicate wells using the MTT assay. Original ODs of cells before conversion to % of control were ≥0.100AU. NT (not tested). *Not Significant (P>0.10).
5.2.8 Molecules that Regulate the Immune Response

5.2.8.1 Introduction

As outlined previously, the bioactivity of products secreted into cell culture media were evaluated by proliferation assay, but this did not reveal information about the specific identity of cell-secreted molecules or those that are expressed intracellularly. This section deals with the molecular basis of this response. Given the results of the study thus far that indicate the cellular responses of the tammar wallaby are similar to eutherian mammals, the investigation of cellular responses was extended to include the identification of key cytokine molecules responsible for the regulation of many of these cellular responses. Optimisation of culture conditions described previously allowed the harvest of RNA from cells that may have expressed cytokines in vitro and the subsequent identification of these molecules using RT-PCR. The successful culture of monocytes in the presence of lymphocyte-conditioned media (Chapter 4) suggested that cytokines produced by both lymphocytes and monocytes might be produced in this culture system. RNA was therefore harvested at times considered optimal for expression of cytokines and transcribed into template cDNA for use in the Polymerase Chain Reaction.
5.2.8.2 RT-PCR Amplification of TNF-α, IL-1β and IL-10 Partial cDNA

Lymphocytes isolated from tammar wallaby lymph nodes (Method 2.3.4.3) were treated with PHA and Con A as described in Section 5.2.6. Supernatants were harvested from these cell cultures and RNA was extracted using Tri-Reagent according to method 2.3.8.1 and cDNA was prepared according to method 2.3.8.2. PCR was performed on this cDNA using the TNF-α primers supplied by Dr. G. A. Harrison according to the protocol outlined in 2.3.8.5 except that an annealing temperature of 55°C was used since these primers had previously been proven on tammar wallaby tissue (Harrison et al., 1999). Single DNA bands of the predicted size were visible after gel electrophoresis of the PCR products (2.3.8.6) from each mitogen, which confirmed the expression of TNF-α by mitogen stimulated tammar wallaby lymph node cells.

Using the same procedures with the exception of a decreased annealing temperature of 45°C to facilitate primer pairing, IL-1β and IL-10 were also amplified from Con A and PHA stimulated lymph node cells (Figures 5.24 and 5.25).

The 387bp *M. eugeni* IL-1β partial cDNA fragment amplified in this study showed 90% identity with that of *T. vulpecula* between the area bounded by the forward and reverse primers (Figure 5.26A). Using the BLASTX program, the deduced amino acid sequence from this fragment was an average 51% identical and 67% similar to IL-1β of eutherian mammals (Appendix Three).

The 198bp *M. eugeni* cDNA fragment identified as IL-10 in this study showed 88% identity with the same region of *T. vulpecula* IL-10 (Figure 5.26B). Using the BLASTX program, the deduced amino acid sequence from this fragment was an average 63% identical and 82% similar to eutherian mammals (Appendix Three).
Figure 5.24: 2% Agarose in TBE gel of IL-1β products. Tammar wallaby IL-1β was successfully sequenced after cloning of the PCR product visualised here with ethidium bromide (arrow). IL-1β cDNA was not detected in potoroo tissues, which is dealt with in Chapter 7.
Figure 5.25: 2% Agarose in TBE gel of IL-10 products. Tammar wallaby IL-10 cDNA was successfully sequenced after cloning of the PCR product visualised here with ethidium bromide (white arrow). IL-10 cDNA was also detected in potoroo tissues, which is discussed in Chapter 7. Strong primer dimers were visible on this gel (black arrow).
A. IL-1β

**ME.** ACCTTCCACAAACTGTGAGCGTTATAGTCTGACTCCAGTCTTGGTGATATCATCCCAAGATGCA
**TV.** ACCTTCCAGAAACTGTGAGCATTATAGTCTGACTCCAGTCTTGGTGATATCATCCCAAGATGCA

**ME.** CCATTCAAGACGCCTCCATCTTCTGCTTCATCGGATGGAATGCTTCTCTCTCCCTTATCT
**TV.** CCATTCAAGACGCCTCCATCTTCTGCTTCATCGGATGGAATGCTTCTCTCTCCCTTATCT

**ME.** CAATGGACAAACACATGAGCCCAAACAAGTGAATTTTCACGCATGAAAGCGGCTCTCCTGGTAAATAGGATCC
**TV.** CAATGGACAAACACATGAGCCCAAACAAGTGAATTTTCACGCATGAAAGCGGCTCTCCTGGTAAATAGGATCC

**ME.** AAGAAAAACACATGGTCTCGCTGTATCAGAAAAATAATCTCTATCTGTCTCTTGTTGGAAGCGTGGCG
**TV.** CAGAAAAACACATGGTCTCGCTGTATCAGAAAAATAATCTCTATCTGTCTCTTGTTGGAAGCGTGGCG

**ME.** AGAAGCCCATCTCCTGCAACTTGAGCAGAATTTGACATTTCTCCACCAATAACGTTGAAAGACAGTTCAT
**TV.** AGAAGCCCATCTCCTGCAACTTGAGCAGAATTTGACATTTCTCCACCAATAACGTTGAAAGACAGTTCAT

**ME.** CTTTACAAGATGGGAATTCAATAATACAACTGAATTTTGAGCCTCGCAGAAATAC
**TV.** CTTTACAAGATGGGAATTCAATAATACAACTGAATTTTGAGCCTCGCAGAAATAC

B. IL-10

**ME.** ATTCAGGTTTACCTGGAGGAAGTGACGCTTGGGCGGAGAAGAATGAGCTGGAATGTCAAAAAGGACG
**TV.** ATTAAGGTTTACCTGGAGGAAGTGACGCTTGGGCGGAGAAGAATGAGCTGGAATGTCAAAAAGGACG

**ME.** TGCCGCTCCCTGAGAAGAAAAGCTGGACTGGGCTCAACACGGTTGTACAGATTCTCCCTCC
**TV.** TGCCGCTCCCTGAGAAGAAAAGCTGGACTGGGCTCAACACGGTTGTACAGATTCTCCCTCC

**ME.** TTGGCGATCCCTAGGAGAAGAAAAGCTGGACTGGGCTCAACACGGTTGTACAGATTCTCCCTCC
**TV.** CTGTGAGGAGCAAACACAGCGGGTTGTCGACAGCAACCTATAAGGAAGCGTCCAGGAAACAA

Figure 5.26: Comparison of IL-1β and IL-10 partial cDNA Sequences from *M. eugenii* (ME) and *T. vulpecula* (TV). Figures (A) and (B) are nucleotide alignments of amplified tammar wallaby IL-1β and IL-10 respectively against the genbank sequences for the brushtail possum (Genbank accession numbers: IL-1β AF071539; IL-10 AF026277). Nucleotides in red highlight the differences between the two species. Amplified regions correspond to nucleotides from the brushtail possum sequence of 392-779 for IL-1β and 317-514 for IL-10.
5.3 Discussion

In this study, optimum conditions for the culture of tammar wallaby lymphocytes were established using QBSF®-51 serum-free media. This medium successfully supported the mitogen stimulation of PBMC, the expression of cytokines TNF-α, IL-1β and IL-10 and the secretion of bioactive factors into culture supernatants. To achieve these results, a number of different experimental strategies were employed. These included:

1. Selection of an optimal cell isolation technique and culture medium after assessment of a selected range of media for the isolation and culture of PBMC
2. Optimisation of the MTT proliferation assay to measure proliferative activity of PBMC
3. Measurement of the proliferation response of PBMC stimulated with plant lectins using the MTT assay
4. Detection of bioactive factors using the MTT assay after their secretion by stimulated PBMC and
5. Detection of the expression of the immunoregulatory molecules TNF-α, IL-1β and IL-10 in cells secreting detectable bioactive factors.

Isolation and Culture of Tammar Wallaby PBMC

Isolation Media

Lymphocytes were successfully isolated from the peripheral blood of the tammar wallaby after trialling a number of protocols and different density gradient media in both 10 and 50mL centrifuge tubes. Percoll, ficoll paque 1.077 and histopaque 1.077 and 1.083 were all trialled with ratios of blood to media of 1:1, 1:2, 1:3 and 1:4. Ficoll-paque density gradient separation with a 1:2 blood/media dilution was effective in both 10mL and 50mL tubes but greater cell recoveries were achieved using a number of isolations in 10mL tubes, rather than the single, larger gradient. Another factor that significantly impacted on the isolation and viability of blood lymphocytes was the exposure to water in the hypotonic lysis step. Strict, short exposures (no longer than 20s) to water followed by removal of haemoglobin as quickly as possible, were necessary in order to sustain cell viability. The use of an ammonium RBC lysis solution in later studies was easier to control and yielded more consistent levels of cell viabilities, especially for recovery of lower cell numbers.
Higher density gradient separation media such as Histopaque 1.083 (H-1.083) used in this study have been used successfully on other mammalian blood. Consistent with findings in tammar wallaby experiments, increased recovery of mononuclear cells was achieved using this medium. However, up to 10% granulocytes were routinely present in these cell fractions. Where H-1.083 was used to isolate *M. eugenii* PBMC, the presence of contaminating granulocytes did not appear to significantly affect the viability of control cells or proliferation results (results from four experiments). The use of this higher density medium is advantageous for recovery of maximal cell numbers from small blood volumes. However, the effect of unidentified factors released by variable numbers of viable, dead or dying granulocytes within the incubation period needs further investigation, since results from the present study were based on a small number of samples where the recovery of monocytes was a priority (see Chapter 4).

Isolation of lymphocytes using percoll gradients (Method 2.3.4.2.1b) resulted in recovery of cells of high purity and viability when blood volumes greater than 10mL were analysed. This procedure has also been used successfully on human cells (Segal *et al.*, 1980) and was advantageous in the present study since the subfractionation of mononuclear cells of a variety of cell densities was achieved. However, the necessity for higher starting cell numbers limited the application of this technique to a small number of comparative studies.

A number of studies using different gradient media report the selective depletion or enrichment of some cell populations in ficoll-paque cell isolation procedures. In a comparative study of ficoll-isolated human lymphocytes against whole blood samples from the same individuals, increases in CD8\(^+\) T cells and decreases in some populations of CD4\(^+\) subsets were reported in cell isolates (Romeu *et al.*, 1992). In marsupial studies, Infante *et al.* (1991) used ficoll-paque to recover mononuclear cells from *M. domestica* and reported a significant loss of macrophages in their preparations. It is possible that some of the variation in stimulation responses between eutherian and marsupial species as well as within the Marsupialia may be explained by these selective isolations. However, these effects were not apparent in the current study, since proliferation results from the small numbers of experiments (n=4) using both ficoll and percoll isolated cells were similar. Percoll isolation should
be considered for future studies if blood volumes greater than or equal to 10mL are consistently available, since this cell isolation method was optimal for larger blood volumes.

Recovery of tammar wallaby blood lymphocytes using ficoll 1.077 isolation was comparable with that of human cell studies where recoveries of approximately 50% of the theoretical lymphocyte yield (usually $10^6$/mL of blood) are routine (Hunt, 1987). In mice and rats, recovery of approximately one third of the lymphocytes with viability of 70-80% from spleen (~$10^8$ cells) and lymph nodes (~$10^7$ cells) is expected using methods employing density gradient media. Mice and rats have smaller lymphoid organs than tammar wallabies and thus splenocyte yields of $10^7$ cells from this species are considered low. This low yield is unlikely to be related to the numbers of lymphocytes within the organ, since histological (Basden et al., 1996) and immunohistological (Hemsley et al., 1995) investigations of tammar wallaby spleen tissue have demonstrated similar lymphoid components to eutherian mammals within this organ. The low yield of lymphocytes from these tissues is more likely due to the harsh conditions employed in the isolation of cells. The presence of high levels of contaminating RBCs and connective tissue debris in spleen cell preparations required repeated washes and ficoll separation steps to remove dead and dying cells (2.3.4.3). Lymph node cells were recovered with higher yields since it was not necessary to pass the suspensions through a sieve as for spleen cell isolations and the viability of these cells was thus maintained. The recovery of $10^9$ cells from two enlarged lymph nodes, two orders of magnitude above the yield from mouse lymph nodes, was higher than expected. However, this result is consistent with the larger size of the nodes when compared with the smaller animals and indicates that the technique used in this study for lymph node cell isolation was successful.

In summary, both percoll and ficoll 1.077 gradient separation media were successfully used to isolate lymphocytes from tammar wallaby tissues. However, larger numbers of starting cells and therefore larger blood volumes were necessary before the percoll isolation protocol could be routinely employed for separation of tammar PBMC. Therefore, ficoll 1.077 separation media was selected as the optimal media for the harvesting of marsupial lymphoid cells since pure mononuclear cell populations were recovered and cells could be isolated from blood volumes as low as 5mL.


**Nylon wool separation**

Cell surface immunoglobulins can increase adherent properties of B cells and thus an incubation on a suitable substrate such as wool fibre can separate lymphocytes into non-adherent T cells and adherent B cells (Trizio and Cudkowicz, 1974). Once isolated from peripheral blood, tammar wallaby lymphocytes were separated into putative T and B cell populations by exploiting differences in their relative adherence to nylon wool fibre. These experiments were not completely successful, although in all cases, the majority of cells were collected within the non-adherent fractions. Limited cell numbers restricted exhaustive optimisation of this technique, but two passages through the nylon wool columns appeared to enrich the non-adherent fraction with cells that were stimulated by PHA. In two experiments of nylon wool separation, apparently elevated levels of adherent cells were present. However, in both of these animals, binucleated cells that may have been plasma cells were identified in the differential white cell counts, which suggests that B cell numbers may have been increased in these samples. Inflated adherent cell numbers may also be due to contamination by residual monocytes that were not completely removed in the preparation of starting cells (Method 2.3.4.2.2). The most obvious explanation for the higher adherent cell proportions is inefficient cell separation through the nylon wool column. This was most clearly the case for cells from animal 592, where visual inspection of both adherent and non-adherent cultures after PHA treatment confirmed a strong agglutination reaction of both lymphocyte fractions. For three other samples, there was an obvious decrease in cell agglutination within the PHA-treated putative B-cell cultures when compared with the non-adherent or putative T-cell fraction.

This technique, though crude, has been used to separate cell populations into non-adherent T and adherent B cells when surface antibodies are unavailable for a number of different species. Nylon wool separation has been used on the lymphocytes of humans (Oppenheim et al., 1968), mice (Handwerger and Schwartz, 1974; Trizio and Cudkowicz, 1974), the grey short-tailed opossum (Infante et al., 1991) and deer (Cross et al., 1996).

Using this technique, lymphocytes isolated from *M. domestica* were identified as adherent, immunoglobulin-positive cells and non-adherent Con A-stimulated cells and
were present in the % ratio of 24 ± 3 : 76 ± 3 (Infante et al., 1991). Whilst the spread of values is higher in the present study (SD=16), the mean recovery of adherent cells for *M. eugenii* was 76% and is consistent with results from *M. domestica* as well as from humans where 70% of circulating lymphocytes are T cells (Hunt, 1987). Future studies focussed on increased incubation times for adherence to nylon wool, changes in the surface area:volume ratio of the column and an increase in the number of passages through the nylon wool, should overcome the inefficiency associated with these separations noted in the present study.

**Selection of an Optimal Cell Culture Medium**

The majority of studies of marsupial immune cell function have used serum-supplemented culture media, specifically foetal calf serum, as a media additive (Ashman et al., 1972; Brozek et al., 1992; Wilkinson et al., 1992b; Buddle et al., 1992; Baker et al., 1998). In the present study, both serum-free and serum-supplemented media were evaluated for their ability to support the culture and mitogen-driven proliferation of tammar wallaby PBMC. Proliferation responses in FBS-supplemented systems were at best variable and mostly reduced when compared with cultures in QBSF®-51 serum-free medium. In agreement with this, Ashman et al. (1976) also reported reduced proliferation activity of PHA-stimulated tammar wallaby blood leukocytes when cells were cultured with media supplemented with FCS or quokka wallaby serum.

**QBSF®-51 Serum-Free Media**

In the present study, optimal responses for tammar wallaby mitogen-driven proliferation were obtained in QBSF®-51 serum-free medium, a commercially prepared low protein (<45mg/mL) medium supplemented with proprietary levels of insulin, transferrin and BSA (Sigma). This media supported the survival of both control and stimulated cells and after five days in culture, up to 50% of control cells remained viable. These results improve upon earlier reports of tammar lymphocytes cultured in FBS supplemented media, where viability was less than 20% after 3 days in culture (Ashman et al., 1976).

The success of tammar lymphocyte culture in QBSF®-51 serum-free media may be attributed to a number of factors. These include consistency of media performance,
compatibility of media with the MTT assay, and media supplementation with additives that supported the metabolic requirements of proliferating cells.

Serum-free culture systems contain defined quantities of biochemical additives that do not vary from batch to batch. In serum-supplemented systems, differences in the growth kinetics of cells may arise due to the presence of both stimulatory and immuno-suppressive growth factors present in different batches of animal serum (Barta et al., 1987; Hügin et al., 1995). This lack of consistency was evident in the present study, where serum-supplemented media was able to support proliferation in some instances but not others when the only change in experimental protocol was different batches of serum.

QBSF®-51 medium enhanced lymphocyte adherent properties that allowed efficient removal of supernatants from culture wells without the loss of low-density cells. In proliferation experiments using the MTT Assay as the detection system, cells cultured in serum-supplemented systems did not sediment well. Others (Carmichael et al., 1987) have also reported problems with floating cell lines when culturing human lymphocytes in experiments using colourimetric proliferation assays. The culture of cells in QBSF®-51 media in the present study successfully overcame this problem due to adherence of cells to the culture vessel.

QBSF®-51 has IMDM as a basal medium and is supplemented with insulin, transferrin and BSA. IMDM contains selenium, pyruvic acid, higher levels of amino acid supplements and twice the level of glucose compared with RPMI-1640. Experiments with serum-supplemented IMDM demonstrated high, unstimulated cell survival with a lower survival rate for stimulated cells (see Table 5.3). The high survival rate of control cells implies that IMDM is a superior culture medium for serum-supplemented cultures of tammar wallaby cells. However, the inability of this media to support proliferation suggests that the serum-free media supplements, insulin, transferrin and BSA, play an important role in cell proliferation.

Insulin and transferrin have been used in a number of different studies to enhance the culture of mammalian cells. Transferrin is present at a high concentration in serum (Guilbert and Iscove, 1976), and most serum-free culture systems that aim to replace
the growth-promoting factors usually provided by serum are augmented with this essential protein. Transferrin promotes uptake of iron by lymphocytes, a co-factor required for the activity of enzymes involved in nucleotide synthesis. Transferrin is thus necessary for the production of new DNA in the S stage of the cell cycle (Herzberg and Smith, 1987). Furthermore, mitogen-driven lymphocyte proliferation is known to be transferrin dependent in eutherian serum-free cultures (Neckers and Cossman, 1983).

In contrast to transferrin, the role of insulin in culture systems is not well understood (Straus, 1984). However, it is known that after mitogen treatments in vitro, the insulin receptor is expressed in rat and human lymphocytes and acts as a marker of B and T cell activation (Helderman and Strom, 1978). Consistent with the present study, murine lymphocytes cultured in insulin-supplemented systems have been shown to proliferate in a manner similar to cells cultured in serum-supplemented systems (Snow et al., 1980). The culture of lymphokine-activated killer cells in a serum-free media that contained transferrin as well as insulin has also been reported (Okamoto et al., 1996).

Measurement of Proliferation

In this study, measurement of cell proliferation was undertaken using the MTT assay. Before the assay was used, a series of experiments were undertaken to validate the method against the more commonly used tritiated thymidine assay. PHA at levels of 25µg/mL and 50µg/mL were used in these preliminary assessments since these levels have previously been used in other marsupial lymphocyte proliferation studies (see Table 5.1).

Proliferation Assays

The most direct measure of increases in cell numbers in culture experiments is to physically count cells. Although time-consuming, this method is feasible when single cell suspensions are the populations under study but more difficult once cell aggregations and colonies have formed after treatment with polyclonal activators. In these situations, cell dissociation treatments are necessary to separate individual cells for counting. These treatments may not always be efficient and the relatively harsh treatment on fragile dividing cells may cause significant reductions in viabilities.
Thus indirect assays that determine cell proliferation are routinely employed to
determine lymphocyte proliferation responses. Two of the most commonly used
indirect techniques include the measurement of the incorporation of radiolabels into
newly synthesised DNA, such as in the tritiated thymidine technique, or the
measurement of biochemical events such as the production of formazan derivatives
after bioreduction by mitochondrial enzymes, such as in the MTT assay.

In this study, the MTT assay was successfully adapted for use on marsupial cells and
there was a direct correlation between numbers of viable cells and mitochondrial
enzyme activity in both standard and suspension cell assays. However, the linearity
of this relationship and the optimum working range of the assay was influenced by a
number of factors. These included numbers of viable cells, detection limits of
spectrophotometric equipment and the ability to dissolve formazan precipitates
formed during reduction of the MTT. Other factors that contributed to the success of
the assay were related to the quality of starting cells, the most important of these
being timely cell separations and efficient removal of RBCs from starting populations
using ammonium lysis.

The proliferation responses of marsupial PBMC after stimulation with PHA were
compared on a selected number of samples using the MTT and tritiated thymidine
incorporation assays. The incorporation of tritiated thymidine into DNA is an
exponential process and lymphocyte proliferation results obtained using the [³H]-
thymidine assay are generally considered to be significant only if stimulation indices
are greater than 3.0 (Kristensen et al., 1982b). Using the MTT assay, OD values
greater than 2 SD over control are generally considered to be an indication of
significant bioactive affects (Yoshimura et al., 1994). In the present study, similar to
other MTT studies, this equates to an SI of >1.20. For tammar wallaby proliferation
experiments, MTT values greater than 1.20 compared well with SI values greater than
3.0 using [³H]-thymidine, suggesting that the MTT technique is an appropriate
alternative for the measurement of marsupial cell proliferation.

Parallel studies were conducted on the same samples for a small number of animals in
this study using both proliferation assays. However, it is difficult to directly compare
these results with those from previous studies since actual direct cell counts are rarely
reported and tritiated thymidine uptake assay conditions are rarely the same. In one study of Con A-stimulated proliferation of human PBMC, actual cell numbers rose from 561 257±75 620 to 1 126 036±155 397 which equates to an SI of 2.0 or a 200% increase in numbers over control cells (Lijnen et al., 1997). This was reported to equate to a SI of between 38 and 91 using [³H]-thymidine incorporation. The control, unstimulated values using this technique varied between 520cpm (from graph) to 2923cpm. Similarly, in a screening study of the in vitro activity of pharmaceutical agents, SIs obtained for PHA stimulation of human cells were similar to those obtained for the tammar wallaby in this study, albeit for lower cell numbers in serum-supplemented media (Yoshimura et al., 1994).

Significant correlations between measurements of mitogen-induced proliferation using the tritiated thymidine technique and the teterazolium-based assays have been obtained for human, mouse and canine lymphocytes (Gieni et al., 1995). Improved sensitivity and decreased standard error of the mean (SEM) between replicate values were also obtained for human and mice cells in these experiments (Gieni et al., 1995). In the present study of tammar cells, this low replicate variability for marsupial blood lymphocytes was confirmed with a standard deviation commonly less than 10% for most results (see Figures 5.6, 5.9 and 5.13).

Reasons for the differences in SI values between these two techniques may be related to a number of properties of the tritiated thymidine incorporation assay. In addition to a measurement of new DNA synthesis, [³H]-thymidine incorporation values may also be a reflection of pre-apoptotic events that involve an increase in nucleotide transporters (El-Metwally and Adrian, 1999). Another factor that may affect these values is the variability in the rate of thymidine kinase activity between different species (Burke et al., 1997). Thus, increased incubation times leading to decreased viability may artificially inflate SI values when using this technique. The use of the MTT colourimetric assay may reduce this effect.

The MTT assay has been used to measure changes in levels of cellular bioactivity (Gerlier and Thomasset, 1986) and as a marker of increased cellular activity associated with the expression of the IL-2 and transferrin lymphocyte receptors (Schauer et al., 1989). It has also been applied to human cell studies for the
measurement of cytokine activity on isolated lymphocytes (Gieni et al., 1995). In the present study, increases in cellular activity not necessarily associated with cell division were measured by this assay, so inspection of cells to confirm increases in cell numbers was undertaken during the optimisation phase of this protocol. Higher numbers of dividing cells were apparent after a 48 hour culture period, so this was chosen as the standard culture time for proliferation experiments. The ability to measure increased cellular activity within short periods of culture is an advantage of this assay that will facilitate future studies of the in vitro functional screening of marsupial cytokines in a defined culture system.

**Mitogen Responses**

Once the MTT Assay protocol was established, a series of experiments investigating proliferation responses of tammar wallaby lymphocytes to mitogens were undertaken. Mitogen-driven lymphocyte responses have been detected in a range of species that include, but are not limited to, guinea pigs (Phillips and Zweiman, 1970), toads (Goldshein and Cohen, 1972), a number of metatherian mammals (Table 5.1), horses, pigs, sheep, humans (Peters and Veerkamp, 1982) and chickens (Lowenthal et al., 1994).

**Mitogens**

A number of commonly used mitogens were assessed for their capacity to cause proliferation of tammar wallaby PBMC.

**PHA**

Levels of PHA between 25 and 50μg/mL were mitogenic for tammar wallaby lymphocytes. These levels are similar to those required for proliferation of lymphocytes of other marsupials such as *M. domestica* (Brozek et al, 1992) and *D. virginiana* (Prasad et al., 1971) and are up to ten times higher than doses of PHA required in eutherian mammals such as humans, mice or rabbits (Brozek et al, 1992). A most obvious, but often overlooked factor that may contribute to differences in mitogen-driven responses between and within species, is the number of different grades of mitogens that are used for in vitro testing. PHA is a mixture of glycoproteins that may vary in mitogenicity from 10 to 100 times dependent on the composition of the particular grade in question (Geppert, 1992). This difference may
provide an explanation to account for some of the factors that have contributed to the variability in reports of *in vitro* mitogenic responses of marsupial cells.

In this study, marsupial lymphocytes cultured in SF media did not display a definite dose-response for this mitogen that was identical for all animals. Fox *et al.* (1976) reported similar findings in a study of opossum proliferative responses. In the present study, levels of PHA between 5 and 100µg/mL caused significant proliferation of tammar lymphocytes. To allow collection of comparative data, PHA at both 25 and 50µg/mL were used for routine experiments since these mitogen levels, whilst not optimal in all cases, consistently caused significant stimulation of tammar wallaby PBMC.

**PWM**

Pokeweed mitogen had the most consistent stimulatory effects in all culture environments tested in this study. Whilst maximal SIs were rarely obtained with this mitogen when compared with either PHA or Con A in both serum and serum-supplemented systems, PWM treatment rarely resulted in reduced responses compared with control cells. Levels greater than 20µg/mL were occasionally cytotoxic for PBMC but concentrations as low as 50ng/mL were stimulatory for this mitogen. This sensitivity prevented the use of PWM as a stimulant for culture supernatant studies since the lower threshold limits for PWM stimulation were not identified and traces of residual mitogen may have influenced bioactive screening studies.

Comparative media experiments with PWM were performed in serum-free and serum-supplemented media and it appears that unlike PHA and Con A, PWM proliferation can be supported in both media systems. The difference in proliferation responses in the two media appeared to be related to timing and the ongoing viability of cells in culture. In serum-supplemented systems, cells remained viable for longer periods. Although activated cells were visible in both media systems after 72 hours, lower numbers of cells were viable in SF media than were present in the serum-supplemented culture.
Con A

Lymphocyte proliferation using this mitogen was best supported in serum-supplemented media (RPMI with 10% low haemoglobin FBS). This finding is similar to the mitogen-driven stimulation results for M. domestica where Con A was a more effective mitogen than either PHA or PWM, when assessed in McCoys media supplemented with 5% FCS (Infante et al. 1991). In the serum-free media used to support PHA-driven stimulation, proliferation results from Con A-treated cells were more variable and SIs of less than 1.0 (MTT assay) were common. Apart from undefined stimulatory factors present in the serum-enriched systems, the comparatively poor proliferation responses of Con A stimulated tammar wallaby cells in SF media appeared to be dependent on the ability of cells to survive in SF culture rather than the lack of response to this mitogen. This effect has also been noted in canine cellular responses, where proliferation of peripheral blood cells was reduced compared with spleen and lymph node lymphocytes isolated from the same animals. This was determined to be due to poor viability of peripheral blood lymphocytes in culture rather than an inherent deficiency in these cells to respond to mitogens (Kristensen et al., 1982a). Very few dose-response experiments were performed with Con A on tammar wallaby cells in the present study. However, where successful, levels of this mitogen between 5 and 25μg/mL were stimulatory for isolated lymphocytes. In both serum-supplemented and SF conditions, large standard deviations were common in proliferation experiments after cells were treated with Con A. This lack of consistency, together with the necessity for culture of Con A stimulated cells in serum-enriched conditions, prevented the use of this mitogen for culture supernatant experiments.

Similar to results obtained for serum-supplemented PBMC proliferation experiments, Con A was routinely stimulatory in whole blood cultures. In summary, the results from this study suggest that Con A is most effective in culture systems that contain unidentified serum components. This may in part be explained by the similarity in three-dimensional structure between Con A and Serum Amyloid P component (SAP), which is a member of a group of animal lectins found in plasma (Ni and Tizard, 1996).
PMA and Ionomycin

Phorbol esters, such as PMA, and calcium ionophores, such as Ionomycin, are known to work synergistically in the activation of eutherian T lymphocytes (Kumagai et al., 1987). When applied to tammar wallaby cells, these two agents independently stimulated proliferation. If these pharmacological substances have similar mechanisms of actions on tammar wallaby lymphocytes as they do on other mammalian cells, the PMA response obtained in this study most likely directly activated protein kinase C. Similarly, Ionomycin is known to promote the movement of calcium ions into the cell cytosol, which is similar to the actions of inositol trisphosphate (Alberts et al., 1989). Both of these responses indicate that a functioning inositol phospholipid signalling mechanism may be present when tammar wallaby lymphocytes are activated.

A dose-response curve was not established for these biochemical agents due to cell number constraints, but experimental data is reported since the strong proliferation responses (% of control between 167 and 273) implies a functional protein kinase C pathway. Whilst an effective cell mitogen, Ionomycin levels required for tammar wallaby cell stimulation were lower than those reported for eutherian cells. A larger study assessing both PMA and Ionomycin responses is necessary before specific conclusions can be drawn, but results in this preliminary study indicate that tammar wallaby cells are able to respond to these compounds in a manner similar to eutherian cells.

LPS

The primary focus of this study was to investigate cell-mediated and innate immune responses. Therefore, proliferation responses using the B cell stimulant, bacterial LPS, were only briefly investigated. However, a small number of experiments were performed to assess the suitability of the SF culture system for assays using this agent. Results from these experiments suggest that bacterial LPS has optimal effect on tammar wallaby lymphocytes at relatively high concentrations (>50μg/mL), which is consistent with the findings for koala blood mononuclear cells (Wilkinson et al., 1992b). LPS responses vary amongst eutherian mammals, and this bacterial factor is not mitogenic for human lymphocytes but does cause proliferation of murine and avian lymphocytes (Nicotra et al., 1985; Weber, 1973). Future dose-response studies
using LPS should focus on optimisation of culture conditions, particularly media type, since LPS is known to interact with a number of serum factors such as LPS-binding protein for its biological effects (Haziot et al., 1993).

Proliferation of Spleen and Lymph Node Cells
Mononuclear cells isolated from tammar wallaby spleens showed proliferation responses to PHA at both 25 and 50μg/mL and decreased responses to Con A at 10μg/mL when cultured in serum-free media. In one animal that was euthanased due to ill-health, the response to PHA was diminished in spleen, but not peripheral blood cells. This finding was similar to that of Ashman et al. (1975) who reported normal PHA dose response behaviour for peripheral blood and lymph node cells from juvenile quokkas, but low spleen cell responses for the same mitogen. Also similar to findings in the present study, Moriarty (1973) found that PHA at levels of 50μg/mL gave optimum mitogenic responses for cells isolated from possum spleens, which was significantly higher than that required for stimulation of rabbit spleen cells at 10μg/mL. Few studies have investigated the in vitro responses of spleen and lymph node cells in adult marsupial species.

Consistent with results for peripheral blood cells in this study, lymphocytes isolated from tammar wallaby lymph nodes, when cultured in SF media, showed optimal proliferation responses with PHA, whilst Con A was the most mitogenic in serum-supplemented media. PWM was also stimulatory for these cells when used at 5μg/mL and demonstrated a slightly reduced response when adherent cells were removed from these cultures. The role of monocytes in the generation of proliferation responses was not studied in detail, but the removal of adherent mononuclear cells in suspension cell preparations routinely diminished stimulated but not control ODs. This suggests that the numbers of monocytes in PBMC preparations may be important in determining the magnitude of in vitro cell-mediated responses.

Interpretation of Findings
Under optimum conditions, SIs for most mitogen driven experiments using the MTT assay were in the range 1.20 – 2.20, although SIs between 1.00 and 1.20 and between 2.20 and 3.20 were sometimes obtained. These results fall within the range reported for mitogen-driven stimulation of human lymphocytes using this assay, where the
maximal proliferation response after treatment with optimal levels of PHA (10μg/mL) was 3.1. Low SIs (<1.0) were not routinely encountered in tammar wallaby assays, but were obtained for a small number of samples. For example, a male animal (1400) that displayed a period of lethargy but survived the viral outbreak that swept through the colony to return to health, displayed a range of SIs between 0.21 and 1.71 at different times during the study. The lowest SIs for this animal were obtained at the time of mating, where SIs dropped from between 1.43-1.71 to 0.21 and 1.05. A one-off sample from a juvenile male (1580) was also taken during a period of mating when it was observed to be competing with the dominant male (1400). The PHA-driven SI for this animal was only 0.53 and he was subsequently transferred out of the yard, where he remained in good health.

Low SIs for two other animals were also obtained in this study. Peripheral blood lymphocytes from the opportunistically obtained animal, 1538, had a SI of 0.88 when tested using PHA. This animal may have been affected by capture stress, since it was not from the original study group and was not accustomed to routine capture techniques. Enlarged lymph nodes evident at necropsy also suggested that 1538 was mounting an immune response at the time of death and it is noteworthy that this animal had previously received reproductive hormone injections (Table 2.1). Animal 209, the original breeding male, was euthanased due to poor condition and found to have a SI of 1.54 for peripheral blood cells in response to PHA. However, this mitogen produced a low SI of 0.61 for isolated spleen cells. In the same round of tests, both PWM (SI=1.64) and Con A (SI=1.69) produced SIs of greater than 1.0 for spleen cells from this animal. These differences in mitogen-driven proliferation responses by lymphocytes from different tissues using the same mitogens and by lymphocytes from the same tissue but using different mitogens suggests that variation exists in the type and/or numbers of lymphocyte mitogen receptors. These differences may be directly related to the state of health of these animals and need to be considered before mitogen responses from a single lymphocyte source are used as an indicator of ill-health.

The mitogen-driven responses of isolated lymphocytes are just one of the parameters that are used to provide an overall picture of the cellular immune response. The total numbers of leukocytes as well as their ratios in peripheral blood have been used as an
indicator of health in humans (Hoffman et al., 2000) as well as a number of other mammals (Schalm et al., 1975). In the present study, there did not appear to be a definite relationship between total cell counts, lymphocyte to neutrophil ratios (L:N) and stimulation indices. The group of animals with total cell counts less than 5 x $10^6$ cells/mL (1400, 1612, 1652, 1844, 8021, 1EF4CB2T and 1E722CFT) contained an animal that was ill and recovered during the study (1400), an animal that died (1652), apparently healthy animals with no offspring (1E722CFT) and those that produced three offspring during the study (1612, 8021 and 1EF4CB2T). Animals 1400, 1652 and 1844 possessed L:N ratios of <1.5 and two of these animals were affected by illness during the study. Total leukocyte counts were not obtained for animals 1008 and 12F5O94T, both of which also died during the study period. Animal 1008 possessed a low L:N ratio of 1.22 and a comparatively high SI of 2.74 as did animal 12F5O94T with the highest SI of the study (SI=3.20) and the lowest L:N ratio of only 0.69. No relationship between total leukocyte numbers, L:N ratio and SIs could be found for animals 202, 6460 and 6693 that also died in this study, although animal 202 had previously tested seropositive to T. gondii, animal 6460 had an injured eye in the months previous to her death and animal 6693 was found at necropsy to be affected by lumpy jaw, an underlying bacterial infection. These factors may have contributed to an underlying immunodepression in these animals and rendered them more susceptible to the viral agent that affected the colony during the course of the study.

Whilst in vitro mitogen testing is not an exact correlate of the in vivo immune response, one advantage of these assays is the ability to stimulate higher numbers of cells and therefore amplify the target response making it easier to study. Lymphocyte stimulation with antigen generally causes the activation of between 0.02 and 0.20% of lymphocytes (Sharon, 1983). In contrast, polyclonal activation with plant lectins may stimulate up to 70-80% of cells in culture, dependent on the lymphocyte subset composition of the test population.

A limitation of this methodology is the high variability between data that is associated with often undefined biological and technical factors (Froebel et al., 1999). To assist with comparisons of results, the effects of this variability can be reduced by expressing results in terms of control values. However, care must be taken that
atyptically low or high values do not unduly influence the final calculated values. A large degree of variability is possible when results are expressed in terms of control responses, since low control values can artificially inflate stimulation indices. Similarly, elevated control cell responses, often associated with immunosuppressed individuals (Rose et al., 1992) can reduce or negate the apparent effect of mitogens. In the present study, only raw data that fell within the working range of the MTT assay was converted to % of control values and unless otherwise stated, OD values below 0.100 absorbance units (AU) were not included in these calculations.

Whole Blood Assays

A technique for the measurement of proliferation responses using whole blood samples was developed in this study. This work was undertaken in order to optimise a method suitable for testing in vitro cell-mediated responses on small blood volumes such as those from young animals and endangered species. Whole blood MTT assays required a great deal of optimisation to balance the numbers of available test cells with the need for reducing interfering levels of RBC. A 1/20 dilution of whole blood in SF media was subsequently found to be optimal for this assay. In contrast to experiments with isolated PBMC, results from MTT, but not tritiated thymidine whole blood experiments suggested that the stimulatory activities of PHA and Con A are reversed in whole blood serum-free experiments and are consistent with the responses of a number of eutherian mammals outlined below. A very small number of assays were performed using the tritiated thymidine assay which may have led to incorrect conclusions, but this difference points again to the importance of culture and detection systems in data interpretation. The presence of serum in whole blood preparations may explain the differential response when using the MTT assay, since Con A was stimulatory in serum-supplemented PBMC experiments. Alternately, the lack of cell isolation steps with density gradient medium in whole blood assays may prevent the selective depletion of Con A responsive lymphocyte subsets that may occur in PBMC preparations.

Whole blood assays using tritiated thymidine detection have been used for the assessment of mitogen proliferation in a variety of different species, including a number of marsupials. Wilkinson et al. (1992b) analysed proliferation responses of koalas over a 3-4 day period using a whole blood dilution of 1:15 in RPMI + 5% FCS
and found that both PHA and Con A stimulation responses were lower than for isolated peripheral blood cells. These researchers also reported the necessity for higher levels of mitogens in these assays, that included Con A levels of between 20 and 80µg/mL, significantly higher than those necessary for the stimulation of tammar wallaby cells in the present study. In another marsupial study, Brozek et al. (1992) found that PHA, Con A and PWM were mitogenic in proliferation assays for M. domestica when whole blood was diluted in Dulbecco’s modified Eagle’s Medium supplemented with 10% FBS.

Whole blood proliferation assays have been used successfully in feline blood at a dilution of 1:20 for 48-72hrs (Tham et al., 1982) where Con A and not PHA had the highest mitogenic effects. Similar to results from tammar experiments, PHA responses for these animals were not significant in whole blood systems. Also similar are results from the whole blood experiments of hybrid striped bass (Morone saxatilis x M. chrysops) where 1/20 dilutions of blood in serum-free medium and cultured for 3 days resulted in high SIs using Con A and PWM but low SIs with PHA (Wang et al., 1997). When used in proliferation experiments on rat whole blood, Con A also caused higher proliferation than PHA at optimal doses of 5 and 10µg/mL when 8µL of whole blood was diluted to 250µL in RPMI with 7.5% FCS and incubated for 72hours.

Advantages of whole blood culture systems include the ability to recover increased cell yields for a given volume of blood since losses in centrifugation steps are avoided and cell populations remain intact since there is no possibility of selective isolation by density gradient media. Whole blood systems also ensure that cultures more closely resemble in vivo conditions since serum factors, platelets and undefined components are still present in the whole blood used for culture. Optimised experiments will be useful for future studies where it is necessary to perform continuous sampling of the same animal for comparative purposes and where small volumes of blood such as for pouch young and endangered species samples are to be tested.

Other Considerations
The diversity of variables examined in the present work prevented a detailed analysis of all factors that may have influenced the in vitro mitogen responses of the tammar
wallaby. However, a number of these parameters are discussed below for their potential to have influenced the mitogen stimulation responses obtained in this study.

**Cell Numbers**

Consistent with other reports of lymphocyte proliferation assays in serum-free media, a higher cell number was necessary to sustain the growth of stimulated cells *in vitro* (Needleman and Weiler, 1981; Blaehr and Ladefoged, 1988). The range of cell numbers that were successfully cultured in the present study equated to stock cell concentrations of $1 \times 10^6$ and $1 \times 10^7$ cells/mL. Whilst this number is similar to levels reported in some PHA-driven marsupial proliferation responses (Brozek *et al*., 1992; Baker *et al*., 1998), cell numbers less than or equal to $10^5$ cells/mL have also been reported in marsupial serum-supplemented culture systems (Ashman *et al*., 1976). In the present study, cell numbers between $10^5$ cells/well and $10^6$ cells/well (optimum 4.5 $\times 10^5$ cells/well) were necessary in order to ensure viability over the 48 hour test period.

The requirement for this comparatively high cell number is not a function of the detection limits of the MTT assay, since cell numbers well below those required for optimal responses were readily detected using this protocol (Figures 5.4 and 5.5). Rather, it is most likely related to the ability of the thymidine assay to measure DNA turnover in surviving cells in an environment where overall cell numbers may be reduced. Hence, cell viability appears to be more critical to the MTT detection method. The requirement for increased cell numbers, together with the tendency of cells in serum-free culture to associate in discrete colonies where cell-cell contact is most likely higher than in serum-supplemented systems, suggests that cell-derived growth factors are also necessary for the metabolic requirements or co-stimulation of marsupial cells *in vitro*. Cell-cell interaction would be optimal in the larger numbers of small cell aggregates formed in the SF medium compared with the fewer, larger colonies formed in serum-supplemented environments (see Figure 5.3). This difference in cell contact may influence the timing of the onset and the levels of production of growth factors that support increased cell growth within the culture system. Certainly in this study, the successful culture of monocyte-derived cells in the lymphocyte-conditioned SF but not the serum-supplemented systems detailed in Chapter 4 confirms that the SF system contains factors not produced, or at least not
produced at the same levels in, the serum-supplemented systems.

Another factor that may have influenced the proliferative properties of female tammar wallaby PBMC is the level of serum prolactin, a polypeptide hormone secreted by the anterior pituitary gland and T lymphocytes. As well as its role in pregnancy and lactation, this molecule upregulates the expression of IL-2 and transferrin receptors on T lymphocytes and promotes the expression of IL-2 itself (Richards et al., 1998; Prystowsky and Clevenger, 1994). In a study of avian lymphocytes by Ibars et al. (1997), a relationship between low SIs and elevated levels of prolactin was demonstrated that was due to a prolactin-promoted increase in base-line control cell activation, which markedly reduced the difference between control and stimulated cell activation. In tammar wallabies, relatively uniform levels of prolactin are present during oestrus and pregnancy and an increase occurs just prior to a birth (Hinds and Tyndale-Biscoe, 1982). However, variations in levels of prolactin are apparent during lactation, with maximal levels usually occurring when pouch young are greater than 100 days old. Since a number of the animals in this study were feeding young, it is possible that some of the variation in SIs recorded throughout the study may be a result of changes in circulating prolactin levels. A more detailed study of the role of prolactin in the immune response should be considered for future studies.

The presence of subclinical or viral infections may also influence the predominant lymphocyte subsets that are available for stimulation using in vitro testing. Experiments conducted on tammar wallabies included testing before, during and after a period when a viral agent was implicated in the loss of approximately 30% of the study colony. Macropod herpesvirus is also known to affect a large number of macropod species (Reddacliff et al., 1997) and no screening for this organism was undertaken prior to this study. In human subjects infected with the human immunodeficiency virus, an early indication of T cell impairment is the loss of proliferative response to PWM (Hofmann et al., 1989; Rose et al., 1992), which indicates a change in lymphocyte subpopulations at times of infection with viral agents. Seropositive, but not clinically ill groups, display this depressed response and it is only clinically ill patients with measurable decreases in their CD4+ T cell counts that display reduced PHA responses. If these generalisations are applied to tammar wallabies tested in the present study, then T lymphocytes were present and functional.
in tammar blood since significant responses to PWM stimulation were apparent in all animals tested.

Differences in mitogen stimulation responses between species may also be due to variations in the distribution and/or expression of lectin receptors on the surface of leukocytes (Sharon, 1983). This area has received little attention in marsupial research, although koala cells were found to bind lectins in different proportions to other animals (Wilkinson et al., 1994). It is possible that cell-surface sugar moieties may differ between the Marsupialia and other mammals, which may explain some of the apparent differences in in vitro mitogen stimulation results reported to date.

Apart from the conditions mentioned above, factors such as age, gender, reproductive status and season were also not investigated in detail for their impact on the stimulation indices achieved for tammar wallaby lymphocytes. Notwithstanding the potential for all of these factors to influence the immune response, most animals in the current study showed SIs greater than 1.0, with the majority greater than 1.2 (usually equal to or greater than 2SD above control cells) when stimulated with mitogen using optimum conditions. Therefore, this technique is useful for the assessment of the general capacity of marsupial lymphocytes to respond to exogenous activation stimuli.

Isolation and Identification of Putative Bioactive Factors

Assessment of Mitogen Stimulated Cell Culture Supernatants

The ability to produce immunoregulatory molecules is clearly an important facet of the cell-mediated immune response. Hence, activated tammar wallaby PBMC were investigated for their ability to secrete bioactive factors that could be identified by their stimulatory or inhibitory actions on the growth of freshly isolated PBMC. Lymphocyte activating factors (LAF) were identified in fractionated culture supernatants enriched for molecules ≤ 50kDa. This crude separation was performed to remove large growth factors and any residual mitogen remaining in the culture fractions. Further fractionating steps were performed to enrich supernatants for molecules <10kDa and for those >10kDa and <50kDa. Whilst large molecules were effectively removed from these preparations, this process most likely did not result in clear-cut separations, since a membrane cut-off of 3-6 times smaller than that required
is necessary for complete exclusion of molecules above the membrane pore size. Therefore, both fractions prepared in this study most likely contained molecules that were less than 100kDa in size and fell within the expected range for most cytokines (Table 5.8).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Molecular Size (kDa)</th>
<th>Principal Cell Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>15-30</td>
<td>Monocytes/macrophage lineage</td>
</tr>
<tr>
<td>IL-2</td>
<td>13-18</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>IL-3</td>
<td>15-25</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>IL-4</td>
<td>15-20</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>IL-8</td>
<td>10</td>
<td>Monocytes, endothelial cells, lymphocytes</td>
</tr>
<tr>
<td>IL-10</td>
<td>37</td>
<td>T &amp; B lymphocytes, monocytes</td>
</tr>
<tr>
<td>Type 1 IFNs</td>
<td>17-26 (monomers)</td>
<td>Lymphocytes, monocytes</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>17-25 (monomeric form)</td>
<td>T lymphocytes, NK cells</td>
</tr>
<tr>
<td></td>
<td>Biologically active form: trimer or tetramers</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>17* (monomeric form)</td>
<td>Monocytes</td>
</tr>
<tr>
<td></td>
<td>Biologically active form: trimers</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8: Molecular Weights of Selected Cytokine Molecules. *Some biological activity (compiled from Smith and Baglioni, 1987; Tizard, 1995; Abbas et al., 1997; Alberts et al., 1989; Mire-Sluis and Thorpe, 1998).

Tammar wallaby mononuclear cells cultured in serum-free media produced mitogenic factors capable of stimulating proliferation and agglutination in freshly isolated PBMC (Figure 5.23). These effects were unlikely to be due to residual mitogen in culture supernatants, since earlier dose-response experiments confirmed that low levels of PHA were not mitogenic for PBMC. Some of these effects were also apparent in supernatants harvested from original control cells, which confirmed earlier experiments where QBSF®-51 media was able to support on-going cell viability and replication. Antiproliferative activity was also identified in low molecular weight fractions of both control and PHA-stimulated lymph node cells obtained from animal 1958 (Figure 5.23).
Upon activation with plant lectins such as Con A and PHA, eutherian T lymphocytes produce IL-2 and IFN-γ, cytokines that are known to promote T cell growth and macrophage activation (Vilcek et al., 1986, Thèze et al., 1996). Given that cells in culture systems stimulated by large molecular weight fractions also contained differentiating monocytes, it is feasible that one or both of these molecules was expressed in the test culture system. To date, molecular studies have failed to detect the gene sequences for either of these two molecules in marsupial genomes (Harrison and Wedlock, 2000), so identification at the functional level is necessary in order to confirm the presence or absence of these pivotal cytokines in the metatherian immune portfolio. Now that a culture system has been developed that supports the production of immunoregulatory factors that can be assessed for their functional properties, experiments to further purify and sequence these molecules can be undertaken.

Whilst the Type II IFN, IFN-γ, may play a role in the proliferation responses of *M. eugenii* lymphocytes in this test system, a Type I IFN, IFN-α, may also be present. IFN-α is known to play a role in the differentiation of PBMCs into dendritic cells (Paquette et al., 1998) and the presence of this molecule in tammar wallaby cell culture supernatants is suggested by the development of monocyte-adherent cells (see Figure 4.4). Further support for the presence of this molecule in culture supernatants is provided by recent molecular studies wherein the genes for IFN-αs has been identified in mitogen stimulated tammar wallaby PBMC (personal communication Dr. G. A. Harrison).

PBMC preparations used for these studies contained lymphocytes, monocytes and cells derived from monocytes. Both IL-10 and TNF-α are produced by cells of the monocyte lineage (Table 5.8) and both of these molecules were identified in the cDNA of mitogen activated cells of the tammar wallaby in this study (5.2.7.2). These two immunoregulatory molecules are known to have anti-proliferative effects on T lymphocytes. Assays using low molecular weight fractions obtained from control and PHA-stimulated lymph node cells from animal 1958 showed these fractions to be inhibitory for PBMC growth. It is possible that these molecules were present and responsible for the inhibitory responses detected in the present study since IL-10 inhibits T lymphocyte responses and TNF-α causes cell cytotoxicity when secreted at appropriate levels (Flick and Gifford, 1984).
The putative identification of cytokine-like molecules secreted by marsupial cells has also been undertaken by Wilkinson et al. (1992b), who identified an IL-2-like factor in the supernatants prepared from cells isolated from the spleen and peripheral blood of koalas. However, the screening test employed in that study involved the maintenance of pre-activated blast cells for a 24-hour period. In the present study of tammar wallaby cells, factors produced by tammar PBMC resulted in significant proliferation responses by freshly isolated PBMCs comparable with the lower range of proliferation values obtained with test mitogens.

The production of an IL-1 type factor in culture supernatants has also been reported for the South American opossum (Brozek and Ley, 1991). The putative IL-1 was produced by LPS stimulated macrophages and skin cells and was detected in a thymocyte co-stimulation assay. This factor, unlike IL-1-like molecules identified in other animals, did not show cross reactivity with other species. This is somewhat surprising since putative IL-1β molecules identified in invertebrate animals show functional reactivity across a number of different species (Raftos et al., 1991). In the present study, IL-1β was identified in the cDNA of mitogen stimulated lymph node cells from the tammar wallaby, so it is possible that IL-1 activity may have been responsible for some of the stimulatory activity apparent on tammar PBMC.

However, before the nature and identity of any of the cytokine-like molecules proposed in this study can be validated, fractions from culture supernatants should be further purified, re-screened for functional activity and isolated for subsequent amino acid sequencing.

Amplification of Partial Cytokine Sequences from Mitogen-Stimulated PBMC

The expression of TNF-α, IL-1β and IL-10 was detected in mitogen stimulated lymph node cells of the tammar wallaby. TNF-α has previously been sequenced in this species after amplification from a mammary associated lymph node (Harrison et al., 1999), but this is the first report of the amplification of both IL-β and IL-10 gene sequences. Given that only partial cDNA products were obtained for each of the IL-1β and IL-10 genes, only a cursory analysis of the sequence information was undertaken, since limited information can be obtained from these relatively small regions. However, it is useful to compare equivalent regions in this animal with a
representative set of eutherian mammals and the only other marsupial sequence available, that of the brushtail possum.

Possum IL-1β putative amino acid sequence alignment against human, murine, rabbit and bovine sequences yielded similarities of between 43 and 46% (Wedlock et al., 1999b), with the most conserved regions being found in the mid and C-terminal region of the protein (residues 186-269). The partial cDNA tammar sequence obtained in the present study was amplified from primers that included this region and is thus expected to have a higher degree of similarity to the possum and other mammals than the entire cDNA region would otherwise have.

IL-10 cDNA amplified from LPS and M. bovis treated alveolar macrophages isolated from the brushtail possum had 52-60% amino acid sequence identity with various eutherian mammals (Wedlock et al., 1998). Again, the partial cDNA product obtained in the present study for tammar wallaby IL-10 was amplified over a region of comparatively high consensus amongst other mammals (between residues 85 and 157 of the mature protein). Thus, this region would also be expected to have slightly higher similarities with other mammals than the entire cDNA sequence for this molecule.

Comparison of sequences from the tammar wallaby with the brushtail possum (Figure 5.26) and other mammals using the BLASTX program (Appendix three), revealed that levels of similarity between each of these animals and eutherian mammals was an average 67% for IL-1β and 82% for IL-10. These values were highly significant (Expected values for highest match: 9.5x10^{-11} for IL-1β and 2.4x10^{-12} for IL-10) when calculated using the BLASTX algorithm, which suggests that these sequences represent tammar equivalents of the cytokines in question. Given that the identity between tammar and possum nucleotide sequences was greater than 85% for partial cDNA regions amplified in this study, it appears that these animals are more similar to each other than to other mammals studied to date as is to be expected based on phylogenetic considerations.

Aside from their primary roles in controlling the host immune response, cytokines such as TNF and IL-1 have been shown to augment the immunological response to
vaccination when used as adjuvants in eutherian mammals (Heath and Playfair, 1992; Rothel et al., 1998). This effect has also recently been demonstrated in the brushtail possum using recombinant forms of TNF-α and IL-1 (Wedlock et al., 1999a and 1999b) and suggests a future role for tammar wallaby immunomodulatory molecules as tools in the prevention of marsupial disease.

5.4 Conclusions
The viability and proliferation of mitogen-stimulated marsupial lymphocytes was assessed in this study using the MTT assay, a colourimetric detection method originally developed by Mosmann (1983) for eutherian cell culture systems. The application of this assay to measure marsupial lymphocyte proliferation was coupled with the culture of these cells in QBSF®-51 serum-free media, a commercially available medium supplemented with proprietary levels of insulin, transferrin and BSA. Using this optimised method, the capacity of marsupial lymphocytes to respond to polyclonal activators such as PHA, Con A and PWM was confirmed. These conditions were subsequently used to produce PBMC-derived immunoregulatory factors that were detected using a slight modification of the serum-free MTT assay. Factors produced during mitogen activation included both stimulatory and inhibitory molecules that were tentatively identified as cytokine-like factors such as IL-2 and members of the IFN family. Anti-proliferative factors were also present in the culture supernatants of control cells grown in untreated QBSF®-51 serum-free media. Preparation of cDNA from RNA extracted from mitogen-stimulated cells was used as a template to detect the expression of TNF-α, IL-1β and IL-10 under serum-free culture conditions. This is the first report of IL-1β and IL-10 sequence for the tammar wallaby and confirms that cells from this animal respond to polyclonal activation in a similar manner to eutherian mammals.
SECTION III

Case Studies

The Long-footed potoroo (*Potorous longipes*)

The Long-nosed potoroo (*Potorous tridactylus*)

The Rufous Hare-wallaby (*Lagorchestes hirsutus*)
Introduction

The Nature of the Study
Whilst little is known about the immune systems of native, endangered species such as the Long-footed potoroo and the Rufous Hare-wallaby, animals from the family Macropodidae are known to be susceptible to both viral and bacterial infections (see 1.4.1). Long-footed potoroos bred in captivity succumb to mycobacterial disease (Phelan, 1996) and although there are no documented reports of mycobacterial infection in the Rufous Hare-wallaby, M. avium infection has also been identified in some animals within a colony of Rufous Hare-wallabies bred in captivity (personal communication Dr. R. McFarlane, Alice Springs Veterinary Clinic). In order to manage these captive animal populations more effectively, there is a need to clarify the factors determining their underlying immune status and hence, the causes for their apparent disease susceptibility.

In this study, the examination of samples collected post-mortem from both healthy animals and those that were suspected to have been overcome by pathological agents provided an opportunity to document the immunopathology resulting from suspected mycobacterial disease. To investigate the functional capacity of immune cells, this work was supplemented with a number of experiments where the capacity of peripheral blood leukocytes to mount both specific and innate responses were assessed.

This section describes the investigation and analysis of samples obtained opportunistically from captively managed populations of endangered small macropod species using standard histological and immunohistological techniques. The identification and functional investigation of peripheral blood leukocytes obtained from these animals were performed using the protocols previously optimised on cells from the model macropodid marsupial, the tammar wallaby (Section II).
CHAPTER SIX
Characterisation of Cells and Tissues

6.1 Introduction
As outlined previously, studies of the immune system of marsupial species have been hampered by the lack of suitable test reagents and protocols and by the limited occasions on which samples of endangered species became available for immunological investigations. Therefore, one of the principal aims of the present work was to collect as much fundamental data as possible when these samples became available. Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby tissues obtained in this study thus provided the opportunity to characterise peripheral blood leukocytes from these three animals. Also, for the first time, the structure and organisation of lymphoid tissues from these animals were documented, with particular reference to the histopathology associated with disease-causing agents such as the Mycobacteriaceae.

6.1.1 Marsupial Peripheral Blood Cells
An overview of the morphology and incidence of marsupial blood cells was presented in Chapter Three, with relevance to cells from the tammar wallaby. This section of the study presents baseline information about the structure and appearance of blood leukocytes from other small wallaby species, the Long-nosed potoroo, the Long-footed potoroo and the Rufous Hare-wallaby. Total leukocyte counts and descriptions of the morphology of peripheral blood cells from the Long-nosed potoroo have previously been reported (Moore and Gillespie, 1968; Parsons et al., 1971). However, there are no published reports of the enumeration or morphology of peripheral blood cells from the Long-footed potoroo or Rufous Hare-wallaby.

6.1.2 Marsupial Lymphoid Tissues
Comprehensive reviews of the histology and immunohistology of marsupial lymphoid tissues have been undertaken by a number of researchers, most notably Canfield and Hemsley (2000) and Old and Deane (2000). In summary, these surveys found that in marsupial species studied to date, the organisation of lymphoid tissues and the type
and distribution of the immune cells within them are similar to eutherian mammals. The histopathology associated with agents of disease in metatherian species is also widely documented (Barker et al., 1963; Munday, 1978; Canfield et al., 1990; Cooke et al., 1995) and it is apparent that marsupial tissue bed reactions to intracellular bacterial and protozoan pathogens often parallel those of immunosuppressed or highly susceptible eutherian species (Canfield et al., 1990; Montali et al., 1998). Of significance to the present study is the metatherian response to mycobacterial agents, which are reported to compromise the health of most marsupial species, particularly those held in captivity (Buddle and Young, 2000).

6.1.3 Mycobacteria

Mycobacterial species belong to the family Mycobacteriaceae, genus Mycobacterium. They are free-living organisms that are known to be pathogenic to mammals (Prescott et al., 1993). Mycobacteria are acid-fast organisms and may exist as curved or straight rods with filamentous morphology. The M. avium bacilli is small (approximately 1.0μm x 0.5μm) and morphologically similar to M. tuberculosis (Thorel et al., 2001). These organisms are aerobic, but are able to survive within host cells since they remain viable in environments of low oxygen tension. Mycobacterial species are catalase positive, a property that may contribute to the neutralisation of reactive oxygen species produced as part of the defence arsenal found within phagocytic cell populations. Virulent strains of this genus possess waxy cell walls (Fenton and Vermeulen, 1996) that also afford some protection from phagocytic digestion by host cells.

6.1.3.1 Marsupial Immune Responses to Mycobacterial Agents - Immunopathology

In eutherian mammals, the marker of a cellular immune response to M. tuberculosis is the formation of granulomas in the lung, which effectively inhibits the spread of these pathogens by isolating the bacteria within these lesions (Anderson, 2001). Granulomas are normally characterised by a macrophage centre containing mycobacteria and a marginal zone comprised of lymphocytes. In most metatherian mammals, experimental infections with mycobacterial antigens have received little attention, with the exception of the brushtail possum, where both experimental and natural infection with the pathogen have been well studied. In possums naturally infected with M. bovis, generalised lesions are common and are often identified in
lungs and lymph nodes (Jackson et al., 1995). In contrast to most eutherian mammals, possums appear to lack the ability to effectively contain mycobacteria within established lesions. This ultimately leads to the formation of satellite lesions when the bacteria spread throughout the host after degradation of the granuloma boundary (Cooke et al., 1995). Granuloma pathology in this species involves central necrosis with large numbers of neutrophils, reduced granulomatous reaction and few, if any, giant cells. Acid-fast bacilli are found in large numbers in necrotic tissue and also in the cytoplasm of macrophages surrounding the areas of necroses.

Other forms of mycobacterial infection, not generally classified in the tuberculosis group, have been identified in a range of marsupial species. As early as 1925, Lucas described the infection of a rat-kangaroo (Aepyprymnus rufescens) after post-mortem results confirmed avian tuberculosis. This animal was found to have congestion in the lungs and miliary abscesses in the liver. Caseous nodules that contained numerous acid-fast bacilli were also identified in the spinal canal of the Bennett’s wallaby (Tilden and Williamson, 1957) with a later report also finding focal necroses in the liver, spleen and various lymph nodes (Hime and Jones, 1972). The spleen and liver of another macropod, the red-necked wallaby (Lesslie, 1958), were also affected by this pathogen.

Atypical mycobacterial disease has been reported in the North American opossum with pathological consequences. Two animals died whilst being held in quarantine, succumbing to mycobacterial disease of the intestines attributed to M. intracellulare (Sevy and Cameron, 1968; Moore et al., 1971). Extensive caseous lesions were found in the mesenteric lymph nodes, spleen and liver of these animals, with histological evidence of proliferative pneumonitis, reduced granulomatous reaction and lesions containing large numbers of acid-fast organisms. Consistent with the response of brushtail possums to tuberculous-type mycobacteria, giant cell formation and fibroplasia were also absent in these animals.

Similarly, atypical mycobacterial infection in a quokka resulted in lesions with a necrotic bacilli-laden core, surrounded by layers of macrophages, epithelioid cells and ‘round’ cells contained within a fibrous outer rim (Munday, 1978). M. intracellulare infection of the brushtail bettong resulted in the development of an ‘intense
pyogranulomatous reaction’ in the joints. Affected areas in these animals presented as zones of macrophages, neutrophils, lymphocytes and plasma cells around central caseous regions (Richardson and Read, 1986).

Bone complications are often associated with mycobacterial disease in captive macropod marsupials (Hime and Jones, 1972) and mycobacterial osteomyelitis has been described in a number of species including the Parma wallaby (M. parma), the Long-nosed potoroo and the tree kangaroo (D. ursinus) (Mann et al., 1982). Granulomas were found in the kidney and liver of the wallaby and lymph nodes contained Langhans‘-type giant cells where caseation had not destroyed the tissue structure. Acid-fast bacteria were found in synovial tissue of the potoroo, together with granulomatous inflammation of the tarsal joint and granulomas in the liver and lungs.

The tammar wallaby and Black-gloved wallaby (M. irma) have also been infected with M. intracellulare (Peet and Dickson, 1982). These infections resulted in caseation of bone material, formation of mineralised lesions that contained acid-fast bacteria and an epithelioid cell response. In another small wallaby, the Long-footed potoroo, animals were found at necropsy to contain lesions in their lungs, liver and spleen due to M. avium-intracellulare. Caseous granulomata with associated giant cells were identified, with acid-fast bacilli located in the granulomata (Phelan, 1996). Similar responses have also been recorded for tree-kangaroos. When affected with M. avium complex, these animals developed granulomatous lymph nodes (Mann et al., 1982) and caseo-necrotic lesions in the liver, lungs, kidneys and bones (Joslin, 1990; Montali et al., 1998).

Whilst there are single reported cases of captive Long-footed and Long-nosed potoroo species being affected by atypical mycobacterial disease (see above), prior to this study, there were no reports of infection of wild-caught animals. Moreover, there are no documented reports of the Rufous Hare-wallaby succumbing to infection caused by mycobacterial agents.
6.2 Results

6.2.1 Identification and Characterisation of the Peripheral Blood Leukocytes of the Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby

6.2.1.1 Separation of Individual Cell Populations
Peripheral blood leukocyte populations were isolated from the blood of the Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby using the protocols previously optimised for tammar wallaby blood leukocyte populations (see Chapters 4 and 5). In most cases, Histopaque 1.083 was used for PBMC isolations. This allowed the maximum numbers of different cell populations to be recovered from a single blood sample (see Chapter 5), although granulocytes were present in numbers up to 10% of the PBMC population.

6.2.1.2 Morphology of Blood Leukocytes
Blood smears were prepared and stained according to Method 2.3.3.1. Only a single sample of Rufous Hare-wallaby peripheral blood leukocytes was processed for electron microscopy. In general, peripheral blood leukocytes from all three small wallaby species were similar to those of the tammar wallaby as previously described (3.2.1).

6.2.1.1.1 Lymphocytes
The Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby possessed small, medium and large lymphocytes that were characterised by their spherical morphology and their higher nucleus to cytoplasm ratio (Figures 6.1Ai, ii, iii, Figure 6.1Bi, ii, iii and Figure 6.2a, b, c). Lymphocytes with indented nuclei were commonly found in blood samples from the Long-footed potoroo (not shown). Azurophilic granules were also apparent in the cytoplasm of a small number of lymphocytes of this species (Figure 6.1Ai). Medium sized lymphocytes of Rufous Hare-wallaby and Long-footed potoroo blood occasionally contained binucleated lymphocytes (Figure 6.2d) and cells with a clear perinuclear staining region, which resemble the plasma cells of eutherian mammals (Figure 6.2b) (Zucker-Franklin et al., 1981). Similar to tammar wallaby cells, the lymphocytes of the Rufous Hare-wallaby contained very few intracytoplasmic organelles and small numbers of mitochondria at the ultrastructural level (not shown). However, different from cells of the tammar
wallaby, the Rufous Hare-wallaby and the Long-nosed potoroo, a proportion of lymphocytes from Long-footed potoroo 880195 were irregular in form, with large diameters and amorphous nuclei that resembled blast cells. This animal had a history of granulomas and these cells were identified in the blood sampled immediately after the animal died.

6.2.1.1.2 Monocytes
Monocytes inspected in blood from the Rufous Hare-wallaby and both species of potoroo typically possessed kidney or horse-shoe shaped nuclei (Figure 6.1Aiv, 6.2e and f). No cytoplasmic granules were visible in any of these cells, although small numbers of cells from each species contained cytoplasmic vacuoles (Figure 6.2e).

6.2.1.1.3 Granulocytes
The neutrophils and eosinophils of the three case study species possessed similar staining properties to the cells of the tammar wallaby. Eosinophilic blast cells similar to those identified in the tammar (3.2.2.1.3) were also detected in the peripheral blood smears of Rufous Hare-wallabies (Figure 6.2h).

Neutrophils
The staining properties, size (Table 6.1) and morphologies of neutrophils were consistent with tammar cells, with neutrophils from all species possessing varied nuclear morphologies. Nuclear lobulation varied between two and seven lobes (Figures 6.1Av, 6.1Biv and v and 6.2i), with 3-, 4- and 5-lobed cells occurring most frequently. Fine lilac-coloured cytoplasmic granules were also visible in these cells when stained with Diff Quik or Giemsa differential stains.

At the ultrastructural level, Rufous Hare-wallaby neutrophils contained Golgi apparati and electron dense granules of various sizes (see Figure 6.3).

Eosinophils
Eosinophils of all three species possessed fine granules that stained dark pink to orange-pink when stained with Giemsa or Diff Quik differential stain. Similar to tammar wallaby cells, granules were densely distributed throughout the cytoplasm (Figures 6.1Avi) and nuclei were typically unilobulated or bilobed. U-shaped nuclei
were common (Figure 6.1Bvi) and some cells contained nuclear lobes that appeared clumped (Figure 6.1Avi).

**Basophils**

Basophils of the Long-footed and Long-nosed potoroo were similar in appearance to those of the tammar wallaby. In contrast, Rufous Hare-wallaby basophils were smaller in average diameter (Table 6.1) than those of the tammar (Table 3.1) and contained both small and large dark blue granules, measuring up to 3μm in diameter (Figure 6.2g).
Figure 6.1: Peripheral Blood Leukocytes of the Long-footed Potoroo and the Long-nosed Potoroo. Peripheral blood smears were stained with Diff Quik differential stain after fixation in methanol. Panel A, representative cells from the Long-footed potoroo. Panel B, white blood cells from the Long-nosed potoroo. A (i) – (iii) and B (i) – (iii) are small, medium and large lymphocytes respectively. The second row of Panel A contains a monocyte (iv), neutrophil (v) and an eosinophil (vi). The second row of Panel B contains neutrophils of distinctly different nuclear morphology (iv) and (v) and a typical eosinophil (vi). Scale bar = 10μm
Figure 6.2: Peripheral Blood Leukocytes of the Rufous Hare-walaby.
Peripheral blood smears were stained with Diff Quik differential stain after fixation in methanol. In the first row, (a) – (c) are small, medium and large lymphocytes respectively. The second row contains a binucleated lymphocyte (d), and two monocytes (e) and (f). The bottom row contains a basophil (g), a blast cell (h) and a pair of neutrophils (i). Note the large granules of the basophil in figure (g). Scale bar = 10μm.
Figure 6.3: Electron Micrographs of the Peripheral Blood Leukocytes of the Rufous Hare-wallaby. Figure (a) is a micrograph of three neutrophils and a blast cell of unknown lineage. Note the multilobed nuclei in all mature neutrophils (M), the Golgi body (G) visible in one cell and the electron-dense heterogeneous granules clearly visible in all of these cells. The blast cell (B) in the centre of field has a few visible mitochondria but no other obvious intracytoplasmic organelles. Magnification x 10400. Figure (b) is most likely a circulating blood monocyte. There are scattered mitochondria and many intracytoplasmic organelles and vesicles (*). RER is clearly visible (arrow). Magnification x 17800. Figure (c) is a high magnification of a section of another blast cell found in this preparation. Note the prominent mitochondria (m) and active nuclear chromatin (e). The nuclear boundary and a nuclear pore are clearly visible (arrow). Magnification x 31 000.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Mean (µm) ± SD</th>
<th>Range (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. longipes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>22</td>
<td>11.7 ± 1.5</td>
<td>9.1 – 14.2</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>21</td>
<td>9.9 ± 2.7</td>
<td>7.1 – 15.5</td>
</tr>
<tr>
<td>Monocyte</td>
<td>7</td>
<td>14.5 ± 1.6</td>
<td>11.5 – 15.9</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>13</td>
<td>11.4 ± 1.5</td>
<td>8.9 – 13.6</td>
</tr>
<tr>
<td><strong>P. tridactylus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>12</td>
<td>12.9 ± 2.1</td>
<td>10.4 – 16.8</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>49</td>
<td>9.5 ± 2.0</td>
<td>7.1 – 15.5</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>5</td>
<td>12.6 ± 1.5</td>
<td>10.6 – 14.2</td>
</tr>
<tr>
<td><strong>L. hirsutus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>22</td>
<td>12.7 ± 0.8</td>
<td>11.2 – 14.2</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>23</td>
<td>12.6 ± 2.2</td>
<td>9.7 – 17.3</td>
</tr>
<tr>
<td>Monocyte</td>
<td>10</td>
<td>15.4 ± 0.8</td>
<td>14.1 – 17.7</td>
</tr>
<tr>
<td>Basophil</td>
<td>4</td>
<td>10.9 ± 2.3</td>
<td>7.1 – 12.4</td>
</tr>
</tbody>
</table>

Table 6.1: Diameters of the Peripheral Blood Leukocytes of the Long-footed Potoroo, the Long-nosed Potoroo and the Rufous Hare-wallaby. Cell diameters were measured using an eyepiece micrometer at 1000x magnification in peripheral blood smears after slides were stained with Diff Quik differential stain.

6.2.1.3 Enumeration of Peripheral Blood Leukocytes

Leukocyte Counts

Differential cell counts were performed on all blood samples obtained from Rufous Hare-wallabies and both species of potoroos according to Method 2.3.3.4. Due to limited sample availability, only two total leukocyte counts were undertaken on any of these samples. When counted using method 2.3.3.3.1, Long-footed potoroos 941208 and 890063 had total leukocyte counts of $8.5 \times 10^6$ cells/mL and $3.3 \times 10^6$ cells/mL respectively, which fall within the reported range for macropod species (see Chapter 3 Discussion). Differential cell counts for the Long-nosed potoroo and the Long-footed potoroo are summarised in Table 6.2 and for the Rufous Hare-wallaby...
are presented in Table 6.3. Levels of eosinophils in Long-footed and Long-nosed potoroos varied within and between animals, but these cells were notably absent from most samples of Rufous Hare-wallaby peripheral blood. This result was unexpected since animals sampled for this study were known to be infected by cestodes and nematodes (autopsy records, Alice Springs Veterinary Clinic). Other noteworthy findings in the differential counts of these animals included the presence of an unidentified blood parasite in Rufous Hare-wallaby 960107 and an increased number of nucleated RBCs in Rufous Hare-wallaby 970518. Band neutrophils and blast cells were identified in many of the blood samples in numbers similar to those in the tammar wallaby.

L:N ratios of <1.5 were considered low for the tammar wallaby in the present study (Chapter 3). However, all but one of the Long-footed potoroos (animal 880195) and two Long-nosed potoroos (animals 990433 and 970690) had L:N ratios of less than 1.5. Similarly, only three Rufous Hare-wallabies possessed L:N ratios that exceeded this value. There was no association between this ratio and clinical health in any of these animals since Long-footed potoroo 880195 had a history of mycobacterial infections, Long-nosed potoroo 990433 had a paternal link to mycobacterioses and Long-nosed potoroo 970690 was in good health. The three Rufous Hare-wallabies with ratios greater than 1.5 (animals 960106, 960107 and 970510) were all apparently healthy and were euthanased for management purposes since animals were over-represented in breeding stock.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Date</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>L:N%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. longipes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>880195</td>
<td>25/11/98</td>
<td>43.3</td>
<td>37.0</td>
<td>11.0</td>
<td>7.1</td>
<td>1.6</td>
<td>54:46</td>
</tr>
<tr>
<td>880195</td>
<td>23/7/99</td>
<td>34.6</td>
<td>60.2</td>
<td>3.2</td>
<td>2.0</td>
<td>ND</td>
<td>36:64</td>
</tr>
<tr>
<td>880195</td>
<td>13/9/99</td>
<td>62.0</td>
<td>34.7</td>
<td>2.5</td>
<td>0.8</td>
<td>ND</td>
<td>64:36</td>
</tr>
<tr>
<td>890063</td>
<td>25/11/98</td>
<td>18.3</td>
<td>69.7</td>
<td>9.2</td>
<td>2.8</td>
<td>ND</td>
<td>21:79</td>
</tr>
<tr>
<td>890063</td>
<td>23/11/00</td>
<td>34.1</td>
<td>58.5</td>
<td>0.0</td>
<td>7.4</td>
<td>ND</td>
<td>37:63</td>
</tr>
<tr>
<td>941208</td>
<td>25/11/98</td>
<td>38.3</td>
<td>52.2</td>
<td>7.8</td>
<td>1.7</td>
<td>ND</td>
<td>42:58</td>
</tr>
<tr>
<td>941208</td>
<td>13/9/99</td>
<td>54.9</td>
<td>43.0</td>
<td>2.1</td>
<td>ND</td>
<td>ND</td>
<td>56:44</td>
</tr>
<tr>
<td>941208</td>
<td>23/11/00</td>
<td>15.5</td>
<td>81.8</td>
<td>0.9</td>
<td>1.8</td>
<td>ND</td>
<td>16:84</td>
</tr>
<tr>
<td><em>P. tridactylus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>950162</td>
<td>25/11/98</td>
<td>26.6</td>
<td>59.3</td>
<td>9.4</td>
<td>3.9</td>
<td>0.8</td>
<td>31:69</td>
</tr>
<tr>
<td>950162</td>
<td>22/7/99</td>
<td>45.1</td>
<td>51.0</td>
<td>1.9</td>
<td>2.0</td>
<td>ND</td>
<td>47:53</td>
</tr>
<tr>
<td>960195</td>
<td>25/11/98</td>
<td>36.4</td>
<td>57.3</td>
<td>4.5</td>
<td>1.8</td>
<td>ND</td>
<td>39:61</td>
</tr>
<tr>
<td>970690</td>
<td>25/11/98</td>
<td>60.8</td>
<td>28.9</td>
<td>8.2</td>
<td>2.1</td>
<td>ND</td>
<td>68:32</td>
</tr>
<tr>
<td>980239</td>
<td>22/7/99</td>
<td>43.3</td>
<td>52.1</td>
<td>0.8</td>
<td>3.6</td>
<td>0.2</td>
<td>45:55</td>
</tr>
<tr>
<td>990433</td>
<td>13/9/99</td>
<td>79.4</td>
<td>16.8</td>
<td>1.9</td>
<td>1.9</td>
<td>ND</td>
<td>83:17</td>
</tr>
</tbody>
</table>

ND not detected

**Table 6.2: Differential Cell Counts of the Long-footed Potoroo and the Long-nosed Potoroo.** Raw data for individual animals are presented here due to the small numbers of samples obtained for each animal. The relationship between lymphocyte (L) and neutrophil (N) numbers is presented as the L:N%.
<table>
<thead>
<tr>
<th>Animal</th>
<th>% Lymphocytes</th>
<th>% Neutrophils</th>
<th>% Monocytes</th>
<th>% Eosinophils</th>
<th>% Basophils</th>
<th>L:N%</th>
</tr>
</thead>
<tbody>
<tr>
<td>238</td>
<td>12.0</td>
<td>77.0</td>
<td>7.0</td>
<td>4</td>
<td>ND</td>
<td>13:87</td>
</tr>
<tr>
<td>241</td>
<td>40.0</td>
<td>58.0</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
<td>41:59</td>
</tr>
<tr>
<td>960106</td>
<td>69.5</td>
<td>22.8</td>
<td>6.6</td>
<td>1.1</td>
<td>ND</td>
<td>75:25</td>
</tr>
<tr>
<td>960107</td>
<td>70.2</td>
<td>25.0</td>
<td>2.4</td>
<td>2.4</td>
<td>ND</td>
<td>74:26</td>
</tr>
<tr>
<td>970500</td>
<td>22.0</td>
<td>74.7</td>
<td>3.3</td>
<td>ND</td>
<td>ND</td>
<td>23:77</td>
</tr>
<tr>
<td>970504</td>
<td>41.8</td>
<td>50.0</td>
<td>7.4</td>
<td>ND</td>
<td>0.8</td>
<td>46:54</td>
</tr>
<tr>
<td>970505</td>
<td>52.5</td>
<td>44.9</td>
<td>2.6</td>
<td>ND</td>
<td>ND</td>
<td>54:46</td>
</tr>
<tr>
<td>970507</td>
<td>23.7</td>
<td>73.4</td>
<td>2.9</td>
<td>ND</td>
<td>ND</td>
<td>24:76</td>
</tr>
<tr>
<td>970510</td>
<td>19.6</td>
<td>77.5</td>
<td>2.9</td>
<td>ND</td>
<td>ND</td>
<td>80:20</td>
</tr>
<tr>
<td>970518</td>
<td>46.4</td>
<td>50.7</td>
<td>2.2</td>
<td>0.7</td>
<td>ND</td>
<td>48:52</td>
</tr>
<tr>
<td>970520</td>
<td>15.4</td>
<td>76.9</td>
<td>7.7</td>
<td>ND</td>
<td>ND</td>
<td>17:83</td>
</tr>
<tr>
<td>980085</td>
<td>16.7</td>
<td>79.6</td>
<td>3.7</td>
<td>ND</td>
<td>ND</td>
<td>17:83</td>
</tr>
<tr>
<td>980202</td>
<td>15.2</td>
<td>81.2</td>
<td>3.6</td>
<td>ND</td>
<td>ND</td>
<td>16:84</td>
</tr>
<tr>
<td>990071</td>
<td>25.4</td>
<td>69.7</td>
<td>4.9</td>
<td>ND</td>
<td>ND</td>
<td>27:73</td>
</tr>
<tr>
<td>ASDP</td>
<td>5.4</td>
<td>89.2</td>
<td>4.5</td>
<td>0.9</td>
<td>ND</td>
<td>6:94</td>
</tr>
</tbody>
</table>

ND not detected.

**Table 6.3: Differential Cell Counts of the Rufous Hare-wallaby.** Raw data for individual animals are presented here due to the small numbers of samples obtained for each animal. The relationship between lymphocyte (L) and neutrophil (N) numbers is presented as the L:N%.
6.2.1.4 Blood Leukocyte Immunocytochemistry

The immunocytochemical slide method optimised on lymphocytes of M. eugenii (see 3.2.2.4.2) was used to identify T cells in isolated mononuclear cell populations of Long-nosed potoroo, Long-footed potoroo and Rufous Hare-wallaby cells. The blood lymphocytes of all three species were positively stained with an antibody directed toward the T lymphocyte surface antigen, CD5 (Table 6.4). The staining of the CD5 antigen was very distinct and intense in the membrane and cytoplasm of a proportion of peripheral blood lymphocytes from all three small wallaby species (Figure 6.4). Similar to the staining of tammar wallaby lymphocytes, staining was optimal when antibodies were used at a dilution of 1/100 and primary antibody was applied for 60mins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Animal Number</th>
<th>Antibody and Dilution</th>
<th>% Positively Stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. longipes lymphocytes</td>
<td>890063</td>
<td>CD5 1/100</td>
<td>67</td>
</tr>
<tr>
<td>P. tridactylus lymphocytes</td>
<td>960995</td>
<td>CD5 1/100</td>
<td>43</td>
</tr>
<tr>
<td>L. hirsutus PBMC</td>
<td>pooled*</td>
<td>CD5 1/100</td>
<td>70</td>
</tr>
</tbody>
</table>

*Pooled sample from Malas 960106 and 990107.

Table 6.4: Immunophenotyping of Mala and Potoroo Peripheral Blood Lymphocytes. Cells were treated with anti-CD5 antibody and visualised with the chromagen, DAB. Data is reported as the mean percentage of CD5 positive cells per field of view (x 1000).
Figure 6.4: Immunocytochemical Staining of the Lymphocytes of the Long-footed Potoroo and the Rufous Hare-wallaby. Long-footed potoroo PBMC (i and ii) and Mala suspension cells (iii) were treated with antibody to the CD5 antigen, visualised here with the chromagen, DAB. Cells were counterstained with Haematoxylin in figures (i) and (ii).
6.2.2 Identification and Characterisation of Tissues of the Long-footed potaroo, Long-nosed potaroo and Rufous Hare-wallaby

6.2.2.1 Detection of Acid-Fast Organisms

Paraffin-embedded tissue sections that included lungs, spleen, lymph nodes, gut, liver, kidney, adrenal glands, brain and skin were stained with Accustain Acid Fast Staining kit for detection of acid-fast bacteria (Method 2.3.6). After washing, blotting and mounting, the acid-fast bacteria stained pink against a green/blue background. A summary of the results obtained from staining a selection of samples of Rufous Hare-wallaby and potaroo tissues is presented in Table 6.5.

In general, acid fast organisms of all three species were localised to histiocytic cells (Figure 6.5), although a number of tissue sections contained bacteria scattered throughout the tissue parenchyma.

*Potoroos*

In addition to being sequestered in macrophages, acid fast organisms of Long-footed potaroo 880195, which had a history of mycobacterioses, were also found in short beaded segments throughout the lung and lymph node parenchyma and in the kidney of wild caught animal 224 (Table 6.5). The lymph nodes of Long-nosed potoroos 990433 and 980239 contained large cytoplasmic bacterial loads, as did the macrophages of the facial lymph node and spleen of animal 950162. These three animals also had a history or association with previous mycobacterial infection (see Table 2.3).

*Rufous Hare-wallaby*

Acid-fast bacteria were detected in a wide variety of anatomical locations in both clinically healthy and diseased animals. Acid fast organisms were detected in the duodenal nodules of animal 960106, the small intestine and lung of animal 241, in a brain lesion of Rufous Hare-wallaby 990068, and in the lymph nodes and spleens of a number of different animals (Table 6.5).
<table>
<thead>
<tr>
<th>Animal</th>
<th>Number</th>
<th>Acid Fast Positive</th>
<th>Acid Fast Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. longipes</em></td>
<td>†224</td>
<td>kidney, spleen</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td>†820054</td>
<td>liver, lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>††880195</td>
<td>lung, lymph node, skin granuloma, spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†910156</td>
<td>lung, lymph node</td>
<td></td>
</tr>
<tr>
<td></td>
<td>990497</td>
<td></td>
<td>muscle, lung, thymus</td>
</tr>
<tr>
<td></td>
<td>224PY*</td>
<td>lymph node</td>
<td>gut</td>
</tr>
<tr>
<td><em>P. tridactylus</em></td>
<td>860257</td>
<td>lung, spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†950162</td>
<td>lymph node, liver, spleen, lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†980239</td>
<td>bone marrow, lymph node, spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>†990433</td>
<td>lymph node, spleen</td>
<td>liver, lung</td>
</tr>
<tr>
<td><em>L. hirsutus</em></td>
<td>†241</td>
<td>gut, lymph node, spleen</td>
<td></td>
</tr>
<tr>
<td>ME960106</td>
<td></td>
<td>duodenum nodules, lymph node</td>
<td></td>
</tr>
<tr>
<td>ME970500</td>
<td></td>
<td>lymph node, spleen</td>
<td>gut</td>
</tr>
<tr>
<td>ME970510</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>†990068</td>
<td></td>
<td>brain plaque</td>
<td></td>
</tr>
<tr>
<td>ME990107</td>
<td></td>
<td></td>
<td>gut</td>
</tr>
<tr>
<td>†89518722</td>
<td></td>
<td>liver, lung, spleen</td>
<td>parotid gland</td>
</tr>
</tbody>
</table>

*PY Pouch Young
† Euthanased due to ill-health
†† Natural Death – history of mycobacterioses
ME Management Euthanasia – surplus to stud requirements

Table 6.5: Summary of the Detection of Acid-Fast Bacteria in Long-nosed Potoroo, Long-footed Potoroo and Mala Tissues.
Figure 6.5: Acid-fast Staining of Mala and Potoroo Tissues. Representative sections from a number of different tissues from each animal were deparaffinised and treated with carbol-fuschin that stained the acid-fast bacteria pink. Tissue sections were counterstained with malachite green. Figure (a) is a section of lung from Long-footed potoroo 880195. The bacteria have been sequestered in the cytoplasm of phagocytic cells (arrows). Similar sequestering of bacteria is also seen in figure (b), a section of lymph node from Long-footed potoroo 910156. A splenic macrophage from a Long-nosed potoroo contained large numbers of acid-fast bacteria (figure c), with organisms also scattered in the surrounding red pulp. Figure (d) is a higher magnification of lung tissue from a Long-footed potoroo. Individual organisms can be differentiated in this micrograph. A section of a duodenum nodule from Mala 960106 is shown in figure (e). Bacteria are clearly seen in the histiocytes of this tissue. Figure (f) is a Langerhans cell of the skin of Long-footed potoroo 880195. Note the almost complete localisation of the bacteria within these cells. Scale bar 25μm.
6.2.2.2 Histology, Immunohistology and Immunopathology of Potoroo and Rufous Hare-wallaby Tissues

Opportunistic samples of potoroo and Rufous Hare-wallaby tissue were processed using routine histological techniques and stained with haematoxylin and eosin (H and E) to allow inspection of tissue pathology (Method 2.3.3.2). Immunohistological investigations using species cross-reactive antibodies to T and B cells were performed to document the distribution of these cells in a representative set of lymphoid tissues. The basic organisation and structure of lymphoid tissues was found to be similar in both species of potoroo and Rufous Hare-wallaby samples, so the results for all animals are presented together.

6.2.2.2.1 Lymph nodes

Lymph nodes from a variety of anatomical locations were inspected. These included mesenteric nodes from all three species, an adrenal-associated node and thyroid-associated node from the Rufous Hare-wallaby and facial nodes from the Long-nosed potoroo.

All lymph nodes were surrounded by a capsule (Figures 6.6a and b; 6.7a and b) with an obvious subcapsular sinus (Figure 6.6e and f), which routinely contained lymphocytes. In all samples, the outer capsule was continuous with the trabeculae (Figure 6.7b), which divided the nodes into discrete regions. The lymphatic exit point, the hilum, was apparent in most sections. Both an outer cortical area and an inner medulla were clearly discernable. The cortex of lymph nodes was composed of primary and secondary lymphoid follicles, trabeculae and lymphatic sinuses. In routine histological inspections, the primary follicles were clearly distinguished by their darkly stained lymphocytes and secondary follicles were recognised by their pale-staining germinal centres and contrasting outer coronas (Figure 6.6a). The medullary regions contained extensive sinuses, medullary cords and obvious blood vessels, all separated by connective tissue (Figure 6.6b and 6.7c).

A paracortical zone that was densely populated with T lymphocytes as demonstrated by anti-CD5 antibody staining (Figure 6.8i and ii), was clearly visible in samples processed for immunohistology. In general, CD5 positive cells were present in large numbers in the paracortical zones and were found in low to medium density in the
lymphoid follicles. These cells were also scattered in low numbers throughout other regions of the nodes.

B cells, evidenced by anti-CD79b antibody staining, were the most numerous cell type found in the cortical follicles (Figure 6.8iv) and were densely collected in the mantle zones of germinal centres (Figure 6.8iii and iv). In general, the germinal centres of secondary follicles were less intensely stained by this antibody and contained lower numbers of CD79b positive cells. Plasma cells, macrophages, lymphoblasts and mitotic cells were present in variable numbers in these regions of the follicles (Figure 6.6d).

Medullary cords contained macrophages (Figure 6.7d) as well as both CD5 and CD79b positive lymphocytes. Scattered throughout the lymph nodes but most prevalent in the cortex, were phagocytic histiocytes, easily identified by their white appearance against a darkly stained background. Long-footed potoroo mesenteric lymph nodes all contained large numbers of these cells (Figure 6.7b).

The general organisation of structural elements was similar for all nodes inspected in this study although there were differences in numbers and presence of primary and secondary follicles and the arrangement of medullary elements.

Atypical Findings
Lymphocytes were present in the lymph node sinuses of a number of different animals. However, as well as lymphocytes, the sinus of the adrenal node of Rufous Hare-wallaby 970500 contained large eosinophilic macrophages (Figure 6.6e). Another uncommon finding was the presence of numerous neutrophils in the subcapsular region of the gut associated lymph node of Rufous Hare-wallaby 241 (Figure 6.6f). Neutrophils were not visible in this region in all other samples inspected in this study.

The medullary region of the facial and mesenteric lymph nodes of Long-nosed potoroo 990433 were extensive and contained prominent dilated sinuses. In the mesenteric lymph node of Rufous Hare-wallaby 241, the paracortical zone occupied an atypically large area of the node and contained predominantly T lymphocytes as
evidenced by immunohistological staining (Figure 6.8ii). CD5 positive cells were densely distributed throughout the node, particularly in the cortical region. B cell follicles were few in number and present at the extreme periphery of the node.
Figure 6.6: Histology of Mala Lymph Nodes (H and E). Figure a) Lymph nodes were surrounded by a thin capsule (arrow) and contained lymphoid follicles (F), some with lighter staining germinal centres, in the cortical regions of the node. Paracortical zones (p) were densely packed with lymphocytes. b) The outer lymph node capsule is more clearly visible in figure (b) (arrow) that also shows the arrangement of the medullary cords (MC) and sinuses (MS). Figure (c) is a lymph nodule embedded in adipose tissue (A) that was found adjacent to the liver of animal 89518722. Small cortical follicles (L) are visible surrounding the more darkly stained medullary region. Figure (d) is a high power magnification of a section of lymphoid follicle showing a germinal centre (GC) with macrophages (m), plasma cells (p) and a mitotic cell (arrow head). A broken line is drawn to indicate the boundary between the GC and the corona (Co). Figure (e) is a photomicrograph of lymphocytes in the subcapsular sinus of an adrenal associated lymph node from animal 970500. Note the eosinophilic macrophage (arrow head). The nodal capsule is visible at top right of field (arrow). In figure (f), an infiltration of neutrophils (arrow) is visible in the subcapsular region of the gut-associated lymph node of animal 960106. Scale Bars: figures (a), (b) and (c) 200μm; figure (d) 10μm; figures (e) and (f) 20μm.
Figure 6.7: Histology of Potoroo Lymph Nodes (H and E). Figure (a). Long-footed potoroo gut associated lymph node with multifollicular cortical and medullary regions. A thin capsule surrounds the node (arrow), which is more visible in micrograph (b). Note the blood vessel visible at bottom left of picture. Figure (b) is a higher magnification of the node showing the connective tissue capsule (Cp), which is continuous with the trabeculae (T). Tingible body macrophages (blue arrows) are scattered throughout the node and are easily recognised by the ‘white’ pallor due to lipid extraction in processing. Figure (c) is a section of Long-nosed potoroo facial lymph node showing medullary cords (MC) and sinuses (MS). Secondary lymphoid follicles (F) are visible in this node, which was surrounded by adipose tissue (A). Figure (d) is a higher magnification of a medullary sinus containing macrophages (m), plasma cells (p) and lymphocytes (L). Scale Bars: figure (a) and (c) 200µm, figure (b) 80µm, figure (d) 10µm.
Figure 6.8: Immunohistological Staining of Long-footed Potoroo and Mala Lymph Nodes. Figures (i), (iii) and (v) are Long-footed potoroo tissue, figures (ii), (iv) and (vi) are Mala tissue. Paraffin sections were stained with anti-CD5 antibody in figures (i) and (ii) and anti-CD79b antibody in figures (iii) and (iv) followed by counterstaining with haematoxylin. Brown staining indicates the presence of the lymphocyte marker. Paracortical zones (P) stained strongly with the T cell marker, anti-CD5. The B-cell marker, anti-CD79b, has strongly stained the periphery of the follicles (arrows) of the potoroo node in figure (ii) and the majority of cells in the follicles of the Mala node. Representative negative control sections are shown in figures (v) and (vi). Scale Bars 200\mu m.
6.2.2.2.2 Spleen

All spleens of the Rufous Hare-wallaby and both species of potoroo were surrounded by a capsule enclosing obvious areas of red and white pulp (Figures 6.9a, b and c). The capsule of older animals was much thicker than those of less mature animals and was composed of fibrous connective tissue as well as a layer of smooth muscle. No distinct cortex or medulla region was apparent. Trabeculae extended inward from the capsule and were diffusely distributed throughout the cellular parenchyma. Vascular (6.9d) and non-vascular trabeculae were evident in cross sections of splenic tissue, with non-vascular types forming the majority of these structures.

The white pulp of the spleen consisted of both splenic corpuscles (lymphatic nodules) (Figure 6.9d) and lymphoid sheaths surrounding arterioles (periarteriolar lymphoid sheaths; PALS) (Figure 6.9e). Lymphatic follicles were distributed throughout the organ and both primary and secondary follicles were identified.

The red pulp of the spleen was composed of splenic sinuses and cords. Erythrocytes, macrophages, plasma cells, lymphocytes, large cells resembling megakaryocytes and myeloid cell precursors were apparent (Figure 6.9f). Endothelial cells were also visible. Venous sinuses were not visible at low magnification but were evident when tissue was inspected using higher magnification.

Immunohistologically, cells of the PALS stained strongly with anti-CD5 antibody, confirming the abundance of T cells in this area (Figure 6.10a). Secondary follicles were stained with this antibody in areas underlying the mantle. Small numbers of T lymphocytes were also scattered throughout the tissue immediately surrounding the PALS. The B-cell antibody stained almost all cells of the primary splenic follicles and densely stained the corona of the germinal centres (Figure 6.10b). Germinal centres of splenic follicles contained sparse numbers of these cells, but also contained lymphoblasts, macrophages, cells with pyknotic nuclei and supporting cells.
Immediately surrounding the white pulp in some Rufous Hare-wallaby spleen sections, was a region of highly cellular red pulp containing large numbers of erythrocytes and leukocytes. This perifollicular zone (Burkett et al., 1993) was obvious in Rufous Hare-wallaby samples 970500, 990109 (Figure 6.9e) and 990107. This region was not apparent in the spleen samples of potoroo species.

**Atypical Findings**

The secondary follicles of Rufous Hare-wallaby 970510 spleen contained very large germinal centres that were unusual when compared with those of the other spleen samples (Figure 6.9d). Splenic sinuses and cords were difficult to differentiate in some of the diseased tissue.

In the animals euthanased due to ill-health, the walls of most blood vessels and venous sinuses appeared to be thickened and in two Rufous Hare-wallaby samples (animals 241 and 990107) and three potoroo samples (Long-nosed potoroo 860257 and Long-footed potoroos 880195 and 910156), extensive fibrosis was apparent. Trabeculae were sometimes difficult to distinguish from granulation tissue. Continuous stretches of white pulp were evident surrounding the arterioles in many of the samples and comprised up to 20% of the splenic parenchyma in most samples. In histological sections of two Rufous Hare-wallaby spleens (970510 and 990068), up to 30% of the total splenic volume was observed as white pulp. This increased amount of lymphoid tissue was associated with extensive lymphocyte infiltration into the surrounding red pulp and marked degradation of the splenic parenchyma.

Granulomas were visible in some of the spleen samples inspected in this study. The spleen of Long-nosed potoroo 950162 was infiltrated with small focal, granulomas that were composed primarily of epithelioid cells, while Rufous Hare-wallaby 241 spleen also contained scattered non-caseating granulomas that contained many plump macrophages (Figure 6.15f). Dense numbers of lymphocytes surrounded the trabeculae in Long-nosed potoroo 950162 tissue as well as in the spleens of Long-footed potoroos 880195 and 910156. In some samples of Rufous Hare-wallaby and potoroo tissue, stromal fibrosis was apparent together with lymphocytic infiltration into the splenic parenchyma.
Figure 6.9: Histology of Mala and Potoroo Spleen (H and E). Figures (a) Mala (990107), (b) Long-footed potoroo (880195) and (c) Long-nosed potoroo (950162) illustrate the basic organisation of splenic tissue. A connective tissue capsule (arrows), which extends into the splenic parenchyma as trabeculae (T), encloses the organ. Areas of white pulp (WP) and red pulp (RP) can be distinguished. Figure (d) is a section of Mala 970510 spleen that possessed large areas of white pulp seen here as secondary follicles containing germinal centres (GC). Lymphoid tissue surrounds a vascular trabecula (VT) and an arteriole (arrow). Figure (e) is a section of parenchyma from Mala 990109. Note the obvious perifollicular zone (pz) between the red and white pulp. Figure (f) is a higher magnification of the red pulp of Mala 990107. Megakaryocytes (Me) are visible amongst the erythrocytes, macrophages (m), plasma cells (p) and endothelial cells (ec) that form the lining of the sinusoids. Scale Bars: (a)-(e) 200μm, (f) 20μm.
Figure 6.10: Immunohistological Staining of Mala Spleen. Figure (a) is a section of Mala 241 spleen stained with a 1/50 dilution of anti-CD5 antibody and visualised with a DAB chromagen (brown). Cells immediately surrounding the arterioles (arrows) are positively labelled. Scattered cells within the adjacent follicles are also CD5 positive. Figure (b) is a section of spleen tissue from the same animal treated with a 1/50 dilution of anti-CD79b antibody. B cells are strongly stained in the primary follicles (F) and the corona (Co) of secondary follicles. Figure (c) is a representative negative control section. All sections were counterstained with haematoxylin (blue). Immunohistological staining of sections shown here was carried out by A. Slender (UWS student supervised by the author). Scale Bars: 200 μm.
6.2.2.3 Gut Associated Lymphoid Tissue (GALT)

Three samples of Rufous Hare-wallaby small intestine were inspected for the presence of GALT. One animal (241) possessed a rim of lymphoid follicles along one side of the gut wall (Figure 6.11a). The remaining two animals possessed normal gut histology. These samples possessed distinct areas of longitudinal and circular muscle adjacent to the submucosa and clearly discernible blood vessels (Figures 6.11b and c). The villi overlying this layer contained a capillary bed surrounded by a surface of columnar epithelial cells and small numbers of goblet cells. Lymphocytes were also identified in the lamina propria of animal 970510 (Figure 6.11c), but both of these gut samples were devoid of obvious lymphoid aggregations.

A Peyer’s patch was identified in the tissues from acid-fast positive animal, 241. In this animal, these lymphoid aggregations were present in the lamina propria, underlying the villi and were covered with a thin layer of epithelium. The Peyer’s patch was composed of follicles with and without germinal centres that were joined by interfollicular areas composed predominantly of T cells as identified by anti-CD5 binding (Figure 6.11e). CD5 positive cells were also detected inside the mantle zone of the germinal centres and in scattered cells of the villi. Strong CD79b B cell staining was evident in the mantle zones of the germinal centres and for almost all cells of the primary lymphoid follicles within the patch (Figure 6.11f). Anti-CD79b antibody did not stain cells in the interfollicular areas and there was no detectable staining of cells with this antibody within the villi.
Figure 6.11: Histology and Immunohistology of Tissue from Mala Small Intestines. Histology. A Peyer's patch (PP) is visible in a cross section of Mala 241 intestine in figure (a). Note the aggregation of lymphoid follicles beneath the villi (V) and overlaying the muscularis externa (ME). Germinal centres are visible (arrows) in some of the follicles. Figure (b) is a section of gut from animal 990107 that has few lymphocytes. The details of the internal structure of the villi (V) and longitudinal and circular layers of the muscularis externa can be distinguished. A capillary (arrow) underlying the lamina propria is also visible. Figure (c) is a gut section from animal 970510 that contained numerous lymphocytes (L) but no organised lymphoid follicles. The crypts of Lieberkühn (CL) are visible in this micrograph. Immunohistology. Figure (d) is a negative control section representative of those obtained for immunohistological staining using both anti-T cell and anti-B cell antibodies. No areas of non-specific staining were evident in gut sections using isotype controls or in the absence of antibodies. Figure (e) shows the staining of CD5 positive lymphocytes in the interfollicular zones (f) of the Peyer’s patch. Scattered cells of the villi (arrow) and a notable number of cells at the periphery of a developing germinal centre are also clearly stained with this antibody. Figure (f) illustrates the almost exclusive staining of the follicular mantle zone with the B cell antibody, anti-CD79b. Immunohistological staining of sections shown here was carried out by A. Slender (UWS). Scale Bars: (a) 350μm (b) 200μm (c), (d), (e) and (f) 100μm.
6.2.2.2.4 Bronchus Associated Lymphoid Tissue (BALT)

Organised regions of lymphoid cells were detected in the Rufous Hare-wallaby and potoroo lung tissue inspected in this study with the exception of the pouch young of Long-footed potoroo 224 that was infiltrated by numerous lymphocytes (Figure 6.12e). Lymphocyte aggregations were located at the periphery of the bronchi and/or between the bronchi and blood vessels (Figures 6.12 and 6.13). Macrophages were also present within these regions as well as in the alveoli and lung parenchyma. A discrete lymphoid nodule adjacent to a bronchus in animal 990433 contained groups of cells resembling mature plasma cells amongst the smaller lymphocytes. Some areas of BALT appeared to contain a developing germinal centre (Figures 6.12d and f).

Atypical Findings

Fibrosis of the bronchial walls was evident in tissue sections from Rufous Hare-wallabies 241, 960106 and 970510. Large multinucleated and epithelioid cells were commonly seen in the interalveolar regions of lung sections from animals 960106 and 241 (Figure 6.13e and f). Foamy macrophages and neutrophils were also identified in the lung tissue of animal 241 along with a small number of focal non-caseating granulomas. The lung parenchyma and some alveoli from Rufous Hare-wallaby 89518722 contained a pink-staining proteinaceous fluid after staining with haematoxylin and eosin. An acellular pale eosinophilic material was also present in the lung parenchyma of Long-nosed potoroo 990433 (Figure 6.12f). Numerous infiltrating erythrocytes and lymphocytes were present and large air spaces resulting from collapsed alveoli were also visible in this sample. Similar cellular infiltrations were visible in the lung section of Rufous Hare-wallaby 990068. However, whilst alveolar volume was reduced due to the presence of increased cell numbers in the interalveolar spaces, the alveoli walls remained intact in this sample.

Congestion (numerous erythrocytes in lung spaces) was evident in Rufous Hare-wallaby samples 970510, 990068 and 960106 (Figures 6.13c-e). Thickened smooth muscle layers were also evident in the bronchial walls of tissue from Rufous Hare-wallaby 241 and Long-nosed potoroo 860257.
Figure 6.12: Histology of Potoroo Lung (H and E). Figure (a) Long-nosed potoroo 860257 with normal lung histology and intact alveoli. A large blood vessel (BV) is proximal to a bronchus (B) at top right of picture. (b) Long-nosed potoroo lung (860257) with a dense lymphocyte aggregate (arrow) between a large blood vessel and bronchus. Lymphocytes are absent from the alveolar spaces (A). Figure (c) Long-footed potoroo 910156 with lymphocytes almost surrounding a large blood vessel (arrows). Figure (d) is a higher magnification of (b). The smooth muscle walls of the bronchus (B) and the adjacent blood vessel (BV) are apparent. Macrophages, with lighter staining cytoplasmas, are visible in the lymph nodule (arrow). Figure (e) is a section of lung from the pouch young of a wild-caught Long-footed potoroo. Note the diffuse lymphocytic infiltrate (L) in the alveolar spaces. Figure (f). Long-nosed potoroo 990433 lung parenchyma containing a dense mat of lymphocytes between the blood vessel and the bronchus. Note the proteinaceous fluid and lymphocyte infiltration present in this tissue. Scale Bars: (a), (c) and (e) 200µm (b), (d) and (f) 100µm.
Figure 6.13: Histology of Mala Lung (H and E). Figure (a) is a section of Mala 241 lung tissue that contains a lymphoid aggregate (arrow) adjacent to a bronchus (B). Alveoli (A) are visible in the surrounding tissue. In figure (b), two lymphoid nodules (arrows) are embedded in the walls of a tertiary bronchus (TB) of Mala 960106. Note the proximity of the large blood vessel (BV). Figure (c) is a section of 970510 lung tissue that was infiltrated with erythrocytes. A collapsed bronchus is visible here, with a lymphoid aggregate at the centre of field. Figure (d) is a section of lung from animal 990068 that also contains many red blood cells and lymphocytes. Despite the obvious tissue pathology, a dense area of lymphocytes is still clearly visible (arrow). Figure (e) is a higher magnification of the lung parenchyma of animals 960106. Erythrocytes, lymphocytes and multinucleated giant cells are evident. Figure (f) is a section of Mala 241 lung that also contains giant cells as well as epithelioid macrophages at right of field (arrows). Scale Bars: (a) and (b) 200μm (c) 100μm (d) 250μm (e) 20μm (f) 25μm.
6.2.2.2.5 Other Non-Lymphoid Tissues

Liver

The histology of the liver specimens from Long-nosed potoroos, Long-footed potoroos and Rufous Hare-wallabies comprised lobules of hepatocytes containing a centriflobular venules and surrounding portal tracts (6.14a and 6.15a). Liver cells and sinusoids radiated from the central vein (Figure 6.14a). Bile ducts were clearly visible and lined by cuboidal epithelium (Figure 6.15a). No areas of haematopoietic activity were apparent in any of the liver sections inspected in this study.

Granulomas were visible in the liver of animal 241 and appeared to arise within the centriflobular venules (Figure 6.14c and d). Fatty change, indicated by areas of white space in processed tissue, was evident in the livers of Rufous Hare-wallaby 889518722 and the Long-nosed potoroo 950162 (Figures 6.14b and 6.15b). A focal granuloma consisting of lymphocytes, macrophages and eosinophils was also detected in 950162 liver tissue (see figure 6.15b and d).

Adrenal Glands

Rufous Hare-wallaby adrenal glands possessed a clearly delineated outer cortex and inner medullary region. The adrenal gland of Rufous Hare-wallaby 990107 appeared to contain a large tumour structure that infiltrated both the cortical and medullary regions. A small isolated focal granuloma that measured approximately 50μm in diameter was present in the adrenal cortex of this tissue. A separate section revealed a larger, neutrophilic cortical lesion of approximately 200μm diameter (Figure 6.14e and f). Neutrophils within this lesion appeared to be suspended in a caseous matrix.

Skin

Granulomas, composed of epithelioid cells and plump macrophages, were found in skin samples of Long-footed potoroos 880195 (Figure 6.15e) and 910156. Plump macrophages and mast cells were also evident in the dermis of some of these sections (not shown). Extensive disorganised lymphocytic infiltration and granulation tissue was present in skin from animal 880195 and collections of lymphocytes were also apparent in a separate, less inflamed section of this tissue. Lymphocytes were particularly visible around small blood vessels. A section of skin from a Long-footed
potoroo showing a lymphatic vessel (LV) and a collection of lymphocytes can be seen in Figure 6.15c.

In summary, histological and immunohistological studies of tissues from the Long-nosed potoroo, Long-footed potoroo and the Rufous Hare-wallaby revealed similar lymphoid tissue structure to other metatherian and eutherian animals studied to date. Where acid-fast bacteria were detected in lymphoid and accessory tissues of these animals, a number of key features were evident in the immunopathological responses. These included the presence of granulation tissue in the skin, spleen and lungs, extensive fibrotic trabeculae within the spleen, lymphocyte infiltrations in the lungs and spleen, the presence of macrophage-rich granulomas in the skin and lungs, a neutrophilic granuloma in the adrenal gland and the presence of multinucleated giant cells in the lungs.

The key histological findings obtained in this study are summarised in Table 6.6.
Figure 6.14: Histology of Mala Liver and Adrenal Gland Lesions (H and E). Normal liver histology is shown in micrograph (a) with lobules of hepatocytes containing a centrilobular venule (V) and surrounding portal tracts (T). Normal hepatocytes (H) surrounded by areas of vacuolation indicative of fatty change (Fc) are visible in the liver of a different animal (889518722) at a higher magnification in figure (b). The liver of animal 241 was scattered with focal granulomas (figure c) which are shown at higher magnification in figure (d). In this micrograph, arrows indicate the boundary between hepatocytes and the cells within the granuloma. Figure (e) is a section of the adrenal cortex of Mala 990107, enclosed by a fibrous capsule (Cp) and containing a granulocytic lesion (arrow). Detail within this lesion is shown at a higher magnification in (f), where individual neutrophils are clearly visible (arrows). Scale Bars (a) and (c) 250μm, (b) and (e) 100μm, (d) and (f) 25μm.
Figure 6.15: Liver, Spleen and Skin Lesions (H and E). Figure (a) is a micrograph showing the normal histology of potoroo liver. A lobule (L) is visible at the top of picture. A portal triad composed of a portal vein (PV), bile ductule (B) and an arteriole (A) is clearly visible in this section. Figure (b) is a section of liver from Long-nosed potoroo 950162 showing fatty change and a focal granuloma (arrow) that appears to be composed predominantly of lymphocytes. Figure (c) is a section of skin granuloma from a Long-footed potoroo showing a lymphatic vessel (LV) and a collection of lymphocytes in the top right of picture (Ly). Figure (d) is higher power magnification of the liver lesion shown in figure (b). Note the bilobed eosinophilic cell (E), the macrophage (M) and lymphocytes (L). A vacuole is also visible (V) at the left of picture. Figure (e) is a skin granuloma (G) from Long-footed potoroo 880195 surrounded by a rim of lymphocytes (Ly). Figure (f) is a section of spleen from Mala 241. Note the similarity in form between the trabeculae (T) and the granuloma (G). Scale bars (a), (b), (e) and (f) 100μm, (c) 25μm (d) 10μm.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Number</th>
<th>AFO**</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. hirsutus</td>
<td>241</td>
<td>+</td>
<td>Gut and neck nodes extensive medullary sinuses, granulomas present in spleen, multinucleated giant cells and BALT in lung, Peyer's patch in gut</td>
</tr>
<tr>
<td></td>
<td>89518722</td>
<td>+</td>
<td>Fatty change in liver, extensive fibrosis and minimal white pulp in spleen with lymphocyte infiltration, BALT present with collapsed alveoli and congestion</td>
</tr>
<tr>
<td></td>
<td>910107</td>
<td>NT</td>
<td>Extensive white pulp and lymphocyte infiltration in spleen</td>
</tr>
<tr>
<td></td>
<td>960106</td>
<td>+</td>
<td>Nodule present in duodenum, white pulp bands and large germinal centres in spleen</td>
</tr>
<tr>
<td></td>
<td>970500</td>
<td>+</td>
<td>Extensive white pulp with germinal centres and clearly defined perifollicular zones in spleen. Degradation of tissue structure with mononuclear cell infiltration</td>
</tr>
<tr>
<td></td>
<td>970510</td>
<td>-</td>
<td>Extensive white pulp in spleen with degraded tissue, some loss of structure and RBC infiltrate, brain plaque</td>
</tr>
<tr>
<td></td>
<td>990068</td>
<td>+</td>
<td>Lymphocyte infiltration of spleen, adrenal gland tumour, granuloma and neutrophilic lesion</td>
</tr>
<tr>
<td></td>
<td>990107</td>
<td>NT</td>
<td>Fibrosis and lymphocyte infiltration of spleen</td>
</tr>
<tr>
<td>P. longipes</td>
<td>224</td>
<td>+</td>
<td>Fibrosis and increased white pulp in spleen. Granulomas and lymphoid nodules present in skin.</td>
</tr>
<tr>
<td></td>
<td>820054</td>
<td>+</td>
<td>Skin granuloma</td>
</tr>
<tr>
<td></td>
<td>880195</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>910156</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>990497</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>224PY**</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P. tridactylus</td>
<td>860257</td>
<td>+</td>
<td>Extensive fibrosis and thickened trabeculae in spleen. BALT in lungs with foamy macrophages in alveoli</td>
</tr>
<tr>
<td></td>
<td>950162</td>
<td>+</td>
<td>Extensive white pulp in spleen with no visible germinal centres, collapsed alveoli in lungs, no GALT</td>
</tr>
<tr>
<td></td>
<td>980239</td>
<td>+</td>
<td>Lymphocyte infiltration and fibrosis of spleen</td>
</tr>
<tr>
<td></td>
<td>990433</td>
<td>+</td>
<td>BALT and oedematous infiltrate in lungs. Lymphocytes in villi of gut tissue.</td>
</tr>
</tbody>
</table>

*Any one tissue tested positive for acid-fast organisms; ** PY Pouch Young; NT Not Tested; †AFO Acid Fast Organisms.

Table 6.6: Summary of Key Histopathological Findings
6.3 Discussion

In the study, the peripheral blood cells and lymphoid tissues of the Long-footed potoroo, the Long-nosed potoroo and the Rufous Hare-wallaby were documented. These cells and tissues were similar to those of the tammar wallaby and other metatherian and eutherian mammals studied to date (Chapter 3 and Old and Deane, 2002b) with the exception of the pathology associated with disease agents. Histological and immunohistological investigations revealed that a host response consistent with attempts to isolate acid-fast organisms within lymphoid tissues was evident in some samples. In others, dissemination of acid-fast organisms to tissues throughout the host appeared to indicate a failed immune response to these pathogens.

I. Characterisation of Leukocytes

(i) Identification and Enumeration of Peripheral Blood Cells

A comprehensive discussion of the incidence and structure of marsupial blood leukocytes was presented in Chapter Three. The size and morphology of peripheral blood lymphocytes, monocytes and granulocytes of the Long-nosed potoroo, the Long-footed potoroo and the Rufous Hare-wallaby were consistent with those of the tammar wallaby (see Chapter 3) and with a previous report for Long-nosed potooros (Moore and Gillespie, 1968). The only notable exceptions to these findings were with the basophils of the Rufous Hare-wallaby, which possessed large, atypical granules and with the large blast-like mononuclear cells detected in the blood of an aged Long-footed potoroo with a history of mycobacterioses, which died from unspecified causes.

In the present study, peripheral blood granulocytes were distinguished from each other by morphology and differential staining characteristics. In Rufous Hare-wallaby blood samples, the identity of basophils was suggested by the dark blue-purple granules and cytoplasm, which is characteristic of these cells (Haen, 1995). The comparatively large size of the granules, whilst atypical for macropod species, is not unusual for the basophils of other mammals such as the guinea pig and the monkey. Basophils of both of these eutherian species possess granules of equal or greater size than their large eosinophilic granules (Schalm et al., 1975). However, confirmation of the identity of these cells should be undertaken in future studies since a subpopulation of neutrophils known as toxic neutrophils (Lewis, 1977) are similar
in morphology to the basophils described in the present study for the Rufous Hare-wallaby. Granulocytes with this description have been described in the peripheral blood of human subjects that have a congenital form of atypical neutrophil (termed Alder-Reilly) and in subjects that have severe infections or burns. Future haematological studies of peripheral blood cells of the Rufous Hare-wallaby should include a biochemical evaluation of granule contents in order to confirm the classification of these atypical cell forms.

Very few total leukocyte counts were performed in the present study due to restricted blood volumes but the two samples that were analysed fell within the normal range for macropod species and were consistent with values obtained for the tammar wallaby (see Chapter 3). In human subjects, lymphocytosis - an increase in total lymphocyte numbers - is associated with chronic infections such as tuberculosis (Lewis, 1977). However, this parameter does not seem to be consistent across all mammalian species since there is no significant difference in absolute lymphocyte counts in normal and mycobacterial infected goats (Caro et al., 2001) and negligible changes in leukocyte and differential counts are reported for mycobacterium affected tree kangaroos (Montali et al., 1998). In the present study, the relative numbers of lymphocytes were high in some of the differential counts recorded for both potoroos and Rufous Hare-wallaby blood (see Tables 6.2 and 6.3). However, true lymphocytosis was not evident since total leukocyte numbers were within the normal range for marsupials (see Chapter 3 Discussion) and fell within the range reported previously for the Long-nosed potoroo (Moore and Gillespie, 1968).

Eosinophilia - the presence of large numbers of eosinophils - is also a condition present in humans affected by mycobacterial diseases such as leprosy and tuberculosis (Haen, 1995). In the present study, levels of eosinophils were elevated (>7.0%) for Long-footed potoroos 880195 and 890063 (see Table 6.2). However, these results were obtained for single sampling events that were not reproduced at later times. It is possible that these high eosinophil levels were associated with an episode of active disease that was not apparent at later samplings. Animal 880195 had a history of mycobacterial disease, which appears to support the finding of eosinophilia in this animal. However, animal 890063 was apparently healthy, although this animal had previously tested positive for M. bovis. No other animal possessed elevated levels of
these cells even though many of the remaining study subjects were found to be acid-fast positive (Table 6.5). The correlation between total and differential cell counts and the presence of mycobacterial disease requires further investigation before assumptions that relate to human subjects can be applied to macropod species.

Similar confirmation is required for comparative leukocyte ratios such as the L:N or N:L ratio, that are commonly used to detect changes in immune status. L:N ratios of less than 1.5 were considered low for the tammar wallaby in this study, although these values are routinely reported for some marsupial species (Spencer and Speare, 1992). In contrast, all but one of the Long-footed potoroos and all but two Long-nosed potoroos had L:N ratios of less than 1.5. Similarly, the ratio of these cells in only three Rufous Hare-wallabies exceeded this value. It appears, at least in small macropod species, that the mean ratio of granulocytes to lymphocytes is not particularly informative. However, results for individual animals suggest that longitudinal monitoring of the changes to these ratios for a single animal may detect baseline changes in leukocyte populations that could prove to be an indication of immune challenge.

(ii) Immunocytochemistry
In this study, immunocytochemical techniques were used to gather baseline information about the presence and numbers of T and B lymphocytes in peripheral blood and tissue samples that were obtained during the course of this study. CD5 positive lymphocytes were detected in the peripheral blood of the Rufous Hare-wallaby, the Long-nosed potoroo and the Long-footed potoroo using a simple immunocytochemical slide technique that was optimised on the cells of the model marsupial, M. eugeni (Young and Deane, 2003). Whilst T cell staining of tammar wallaby lymphocytes was variable (see Chapter 3 Discussion), the values obtained for CD5-positive peripheral blood lymphocytes of Rufous Hare-wallaby and both potoroos were consistent with the levels of CD3 positive cells detected in the blood of another marsupial, the koala (Wilkinson et al., 1995). These results also fell within the range reported for CD3 positive lymphocytes in lymphocyte smears of eutherian mammals such as dogs (55.1 ± 3%), pigs (56.4 ± 2%), sheep (51.8 ± 5%) and cattle (49.3 ± 6%) (Levkutova and Revajova, 1997), which suggests that these species
possess similar numbers of circulating T cells to other more common mammalian species.

The nature of the intracytoplasmic staining used in this assay limits the usefulness of these antibodies to identification studies rather than as a protocol for labelling viable cells after separation. However, a major advantage of the slide technique is that small blood samples such as those obtained from endangered or young animals can be analysed with ease. This is particularly pertinent to the analysis of rare samples such as those obtained in the present study, since the visualisation of the location and distribution of antigen within the test cells using microscopic techniques can be performed at the same time as the determination of the purity of the isolated cell preparation. This procedure may also be used for diagnostic purposes once a registry of the normal range of lymphocyte phenotypes is established.

II. Characterisation of Tissues

In this study, tissue samples obtained from the Rufous Hare-wallaby and the Long-footed and Long-nosed potoroo were investigated for evidence of the capacity to mount an immune response against intracellular bacteria such as the Mycobacteriaceae. To this end, histological sections were inspected for the presence of acid-fast organisms as well as for the basic organisation of lymphoid tissues. Immunohistological investigations were also performed on representative samples to determine the distribution of lymphocyte populations within these tissues.

(i) Detection of Acid-fast bacteria

Examination of the tissues of Rufous Hare-wallaby and potoroo species for acid-fast bacteria was undertaken in an effort to confirm the preliminary diagnosis of mycobacterial disease where applicable and to provide an explanation for the general pathology evident in some of these tissues. Small organisms consistent in size and general morphology with M. avium were present in acid-fast positive tissues of the Rufous Hare-wallaby, the Long-nosed potoroo and the Long-footed potoroo. These organisms were generally localised to phagocytes within the lymph nodes, lungs, spleen and skin although they were also detected in the liver and kidney of one animal and were scattered throughout the tissues of the most severely affected hosts. M. avium-intracellulare has previously been detected in the potoroo colony (Phelan,
1996) and *M. avium* has been identified post-mortem in an animal from the Rufous Hare-wallaby Desert Park group (personal communication Dr. R. McFarlane). It is therefore likely that the organisms detected in the present study were also from this genus.

The detection of acid fast organisms in Long-footed potoroo 224 is believed to be the first report of an acid-fast positive wild-caught Long-footed potoroo. Also of significance in this study was the finding that the Long-nosed potoroos, presumed to be largely unaffected by mycobacterial disease before commencement of this work, were almost all infected by acid-fast organisms (see Table 6.5). Furthermore, a number of Rufous Hare-wallaby samples provided for ‘control’ comparisons were also found to contain acid-fast organisms and it remains unclear whether this pathogen was endemic in this colony or present as an opportunistic pathogen.

(ii) Immunohistology

Previous immunohistological studies on the macropod species *M. eugenii* (Hemsley *et al.*, 1995; Old and Deane, 2002b) and *M. giganteus* (Old and Deane, 2001) using anti-CD5 and CD79b antibodies, confirmed the suitability of these test reagents as T and B lymphocyte markers for the small wallaby species investigated in the present work. The selection of an anti-CD5 antibody rather than the commercially available anti-CD3 antibody was due to the clarity of staining obtained with this reagent. Anti-CD5 also lacked the background staining that is sometimes associated with the anti-CD3 antibody (Hemsley *et al.*, 1995). The anti-CD79b antibody was selected since marsupial lymphocytes are consistently stained with this B cell marker (Jones *et al.*, 1993; Hemsley *et al.*, 1995; Baker *et al.*, 1999; Old and Deane, 2001).

In the present study, both T and B cell antibodies consistently recognised areas of lymphoid tissue that correspond with T and B lymphocyte regions of other marsupials (Hemsley *et al.*, 1995; Old and Deane, 2001 and 2002b). However, there have been two reports that suggest that a small population of B cells may also be recognised by the antibody to the pan T cell marker CD5. In lymph nodes of the northern brown bandicoot, small numbers of cells with histology resembling plasma cells were positively labelled with anti-CD5 antibody (Cisternas and Armati, 2000). In another marsupial, the Eastern grey kangaroo, larger numbers of follicular lymphocytes within
GALT were stained with anti-CD5 antibody than with the pan T-cell antibody, anti-CD3 (Old and Deane, 2001). This suggests that a different population of cells was labelled with anti-CD5 or at least possessed this cell marker in addition to the CD3 antigen. Since CD5 is co-expressed on the surface of B cells in some species (Kipps, 1989), it is possible that a small number of potoroo and Rufous Hare-wallaby B cells also co-express this antigen. Notwithstanding the potential for low numbers of cross-reactive cells, the obvious differences in distribution of CD5 and CD79b positive lymphocytes detected in the lymphoid tissues of small wallaby species in the present study suggests that these two antibodies are useful for the gross localisation of T and B lymphocytes within macropod tissue beds.

(iii) Histology and Immunopathology
After initial entry into the host, the *Mycobacteria* are generally presented by macrophages to lymphoid cells in regions such as the BALT, GALT and within lymph nodes that drain these sites (Lugton, 1999; Thorel *et al.*, 2001). If the bacteria are not contained within these regions, they will quickly spread via the lymphatic drainage systems to the bloodstream and eventually colonise the liver, kidneys and spleen (Collins and Campbell, 1982). In this study of marsupial immune responses, it was therefore important to document the histopathology of these organs in order to understand the effectiveness of the host response to mycobacterial infection.

Lymphoid Tissues

a. Lymph nodes
The basic organisation of lymph nodes in all animals included clearly demarked cortical and medullary regions enclosed by a fibrous capsule. Lymphoid follicles were situated in the outer regions of the node and were characterised by lighter staining germinal centres when stained with H and E. This organisation is similar to that described for other marsupials such as the northern brown bandicoot (Cisternas and Armati, 1999) and two macropod species, the quokka (Ashman and Papadimitriou, 1975) and the tammar wallaby (Basden *et al.*, 1997), as well as most eutherian mammals (Banks, 1981; van der Valk and Meijer, 1987) with the exception of the pig, where the structure is inverted (Spalding and Heath, 1986; Binns and Pabst, 1994).
Small lymphoid nodules were found in the skin of Long-footed potoroo 880195 and embedded in the adipose tissue adjacent to the liver of Rufous Hare-wallaby 89578722. These tissues appeared to be unencapsulated, were structurally less complex than larger lymph nodes, and contained discrete regions of densely packed lymphocytes that were interspersed with macrophages (Figure 6.6c). When compared with the lymph nodes of other species, these lymphoid nodules appear to share some similarity in structure to the lymphoid regions of both birds and monotremes. Although most avian species do not possess true lymph nodes, they do possess discrete arrangements of lymphoid cells that surround lymphatic vessels and form secondary follicles (Banks, 1981; Tizard, 2000). Similar to the lymphoid nodules of the potoroo and Rufous Hare-wallaby, these structures also do not possess an obvious capsule. The architecture of monotreme lymph nodes is also less complex than in higher mammals and the lymphoid nodules identified in the present study shared some structural similarities to those described for the echidna (Diener and Ealey, 1965). In this species, the comparatively simple arrangement of lymphoid tissue was assumed to result in less complex immune function capacity and hence was linked to the production of reduced secondary antibody responses in this species. Analogously, the presence of both small, simple lymphoid nodules and larger, more complex lymph node structures in macropod species may lead to a spectrum of immune responses depending on the site of macropod species may lead to a spectrum of immune responses depending on the site of antigen presentation. In part, this may account for the sluggish or delayed secondary responses reported for a number of marsupial species (Wilkinson et al., 1992a). Further studies designed to identify and characterise the function of these nodules should be performed to assess the validity of this hypothesis.

In eutherian mammals, the lymphocyte-dense follicles within lymph nodes are generally rich in B cells that may survive for up to seven weeks in the tissue beds (Fulcher and Basten, 1997). Germinal centres are formed within these follicles in response to antigenic challenge and are indicative of secondary responses such as lymphocyte proliferation and antibody production (Ross et al., 1995). Secondary follicles were visible in all nodes inspected in this study, although their numbers and sizes varied from animal to animal and the numbers and types of cells within the germinal centres also differed. Plasma cells, macrophages and mitoses (Figure 6.2) were all present in these regions, as were epithelial cells and stromal elements. The
presence of active germinal centres within the lymph nodes of all animals investigated in this study suggests that at the time of tissue collection, a humoral immune response was in progress within these nodes. Whether or not this response was generated as a result of exposure to mycobacterial antigens remains unclear, however humoral immune responses are known to be ineffective in the resolution of mycobacterial disease (Caro et al., 2001).

Immunohistological staining of lymph nodes revealed that T cells formed the major cell population in the paracortex, which is consistent with the lymphocyte arrangement in tammar wallaby lymph nodes (Old and Deane, 2002b) and with other marsupial species (Hemsley et al. 1995). The mantle of the secondary follicles contained predominantly B lymphocytes, while CD5 positive cells, presumably T helper cells since B cell activation by T cells is necessary for the formation of germinal centres (Kelsoe, 1996), were also found in low numbers within both primary and secondary lymphoid follicles. CD5 staining in the coronas of cortical lymph node follicles identifies the presence of T helper cells in these regions in humans and similar arrangements of these cells in macropod lymph nodes suggests that these cells have a similar function in the tammar wallaby (Old and Deane, 2002b), the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo.

In eutherian mammals, the arrangement and numbers of follicles in the cortex and the depth of the paracortical region may vary according to the immune status of the individual lymph node and the organism as a whole (Poppema et al., 1981; van der Valk and Meijer, 1987). A predominantly cytotoxic immunological response is associated with paracortical thickening whereas a primarily humoral response is taking place when many cortical follicles with pale germinal centres are present (Burkitt et al., 1997). In the present study, the paracortex of the gut associated lymph node (GALN) of Rufous Hare-wallaby 241 occupied a much larger area than the same regions in either of the GALNs isolated from Long-footed (Figure 6.4) or Long-nosed potoroos. Few B cell follicles were present in the Rufous Hare-wallaby node. A predominantly cell-mediated response called paracortical hyperplasia is mounted by humans in response to viral agents and is characterised by high numbers of parafollicular T lymphocytes that appear to ‘push’ small B cell follicles to the periphery of the node (Burkitt et al., 1997). The nodal architecture of Rufous Hare-
wallaby 241 resembled this arrangement and is consistent with reports of the presence of an orbivirus within the Rufous Hare-wallaby colony (personal communication, Dr. Ro McFarlane).

In contrast, the Long-footed potoroo (910156) GALN was composed predominantly of lymphoid follicles, a comparatively small paracortex and little medullary region (Figure 6.3a). As previously outlined, this arrangement is consistent with a B cell, humoral response. Long-nosed potoroo and Rufous Hare-wallaby mesenteric and face/neck nodes contained extensive medullary sinuses, different from Long-footed potoroo lymph nodes, which possessed normal medullary organisation. Expansive sinus areas within medullary regions of lymph nodes are common in areas where the node is situated at a common drainage point such as in the submaxillary region (Gartner and Hiatt, 1994), so it is unlikely that these large sinus areas were pathological in nature.

Histopathology
In general, the structure of the lymph nodes inspected in this study was intact, with no evidence of caseous necrosis that is usually associated with advanced cases of mycobacterial infection in marsupials and other mammals (Cooke et al., 1995; Burkitt et al., 1997). Clearly demarked granulomas were not apparent in any lymph node inspected in this study. However, neutrophils were present in the subcapsular region of the lymph node of acid-fast animal 960106, which suggest that an early granulomatous response may have been occurring in this node.

Atypical mycobacterial infection usually involves the lymph nodes and where generalised disease is evident, pathology may also include the liver and spleen (Thorel et al., 2001). Unlike infections with M. tuberculosis, caseous necrosis of the lymph node is rare and giant cells are sparse. In immunodeficient hosts, histological inspection of the lymph node reveals aggregates of epithelioid cells and/or foamy macrophages with very few, if any lymphocytic cells (Burkitt et al., 1997). This form of response was not evident in any of the lymph nodes inspected in this study, which suggests either that animals were not immunodeficient since they were controlling the infection, or that they were not in a very advanced stage of mycobacterial disease. It is also possible that there were more severely affected lymph nodes which were not
sampled. The latter proposition is unlikely, since mesenteric, cervical and submaxillary nodes were inspected from a number of different animals and these nodal sites are usually implicated in this form of disease.

b. Spleen

The general organisation of the Rufous Hare-wallaby and potoroo spleens was similar to eutherian mammals (Banks, 1981; Gartner and Hiatt, 1994; Tizard, 2000) with clearly distinguishable regions of white and red pulp. Rufous Hare-wallaby and Long-footed potoroo samples also contained numerous trabeculae, extending from the tissue capsule into the splenic parenchyma. Variations in the histology of normal spleen tissue exist both between species and within individuals in a species (Banks, 1981; Burkitt et al., 1993) and some of these differences were evident in the present study. Unlike human and bovid species but similar to those of the horse, dog and cat, the Rufous Hare-wallaby and potoroo spleens contained extensive trabeculae that supported numerous penicillar arterioles. This finding is consistent with observations by Hayes (1968) and later by Cutts and Krause (1982) for another marsupial, the Virginia opossum (Didelphis virginiana) and also some eutherian mammals, where the spleen functions as a blood storage organ (Banks, 1981). Megakaryocytes were also present in Rufous Hare-wallaby and potoroo spleens (Figure 6.5f), which is similar to findings for a variety of mammals such as rodents, felines and monotremes (Banks, 1981; Connolly et al. 1999) and suggests that platelet storage may be a function of this organ in macropods.

The regions of white pulp within the spleens of the Rufous Hare-wallaby, the Long-nosed potoroo and the Long-footed potoroo all conformed to the structural arrangements previously described for macropod and eutherian mammals (Canfield and Hemsley, 2000; Old and Deane, 2000). Dense lymphoid tissue immediately surrounded prominent arterioles as periarteriolar lymphoid sheaths (PALS) and was composed primarily of CD5 positive T lymphocytes. B lymphocytes that stained positively with the CD79b antibody were mainly located in the corona of secondary follicles that were adjacent to the PALS. Primary lymphoid follicles were recognised in white pulp regions by their lack of germinal centres and B cell staining of the majority of cells within the follicle.
A blood congested region between the germinal centres and mantle regions of splenic follicles was apparent in tissue from Rufous Hare-wallabies less than two years of age but was not evident in other Mala samples or tissue from either of the potoroo species. This obvious perifollicular region has also been described in platypus spleen (Connolly et al., 1999) but has not been described for any other marsupial species studied to date. Given that this area was not identified in any of the adult tissues in the present study and that both of the young animals were sacrificed for management purposes not associated with obvious disease, it is possible that this blood-filled zone represents a stage in the maturity of the splenic structure and is not related to any immunopathological event.

**Histopathology**

In some Rufous Hare-wallaby and Long-footed potoroo tissue, trabeculae were thickened and fibrotic but in others, were difficult to distinguish from lesions. The degree of fibrosis in these tissues was most likely associated with an immunological response since fibrous and thickened trabeculae were also described in spleens of koalas affected by pneumonia, hepatitis and other ill-defined diseases (Backhouse and Bolliger, 1961). As well as this fibrosis, large areas of white pulp were visible in two Rufous Hare-wallaby spleens that were positive for the presence of acid-fast bacteria. These tissues contained numerous secondary follicles adjacent to arterioles, atypically large germinal centres were apparent and extensive lymphocyte infiltration and general splenic disorganisation characterised these tissues. In other mammals, variation in the numbers and types of lymphoid follicles is an indicator of the immunological activity of lymphoid tissue (Kelsoe, 1996). Thus, whilst variations exist in the ratio of red pulp to white pulp in most mammalian species, increased levels of white pulp in the spleens of some animals in this study correlated with general pathology and thus was most likely the result of disease.

In eutherian mammals, one of the primary functions of the spleen is to remove foreign matter, including bacteria, from blood flowing through the venous sinuses (Banks, 1981; Tizard, 2000). The spleens of acid-fast animals in this study all demonstrated histopathological responses consistent with the presence of a pathogen carried by the circulatory route. Thickened blood vessel walls, extensive areas of white pulp surrounding arterioles, lymphocyte infiltration and the sequestering of acid-fast
organisms by macrophages were all indications that the spleens of small macropod species play a major role in the eradication and clearance of antigen.

c. Gut Associated Lymphoid Tissue (GALT)
GALT includes organised regions of lymphoid tissue such as the Peyer’s patches and mesenteric lymph nodes as well as discrete lymphoid cells that are scattered amongst the gut tissues. Atypical mycobacterial infection is known to involve the alimentary tract of mammals (Thorel et al., 2001), and this tissue plays an important role in the ability of the intracellular bacterium to enter the host. M cells and follicle-associated epithelia appear to facilitate the access, transport and excretion of M. bovis in a number of species (Lugton, 1999). Similarly, M. avium is reported to gain entry to the host via endocytosis by M cells, intra-epithelial lymphocytes and antigen presenting cells as part of the normal functional host immune response.

Three samples of Rufous Hare-wallaby intestine were inspected for immunopathology in this study; one from an acid-fast positive animal (241) and two from acid-fast negative animals (970510 and 990107). No samples of potoroo gut were available for inspection. Histologically, the intestines of the Rufous Hare-wallaby possessed normal structure and no obvious immunopathology was apparent. However, a series of lymphoid follicles (Peyer’s patch) was identified in the section of Rufous Hare-wallaby 241 intestine. Immunohistologically, the follicles of the patch stained strongly for B cells and contained small numbers of T lymphocytes in the corona of the follicles. In eutherian mammals, the Peyer’s patches of the gut contain the highest fraction of B lymphocytes, where they comprise approximately 70% of the total lymphocyte population (Hunt, 1987). Also similar to human samples, CD5 positive T lymphocytes were located in the lamina propria, the intestinal intraepithelium and the interfollicular zones within the patch.

Whilst gut lymphoid aggregations have been described in a number of Australian marsupial species such as marsupial mice, Antechinus swainsonii and A. stuartii (Poskitt et al., 1984), koalas (Hanger and Heath, 1994; Hemsley et al., 1996), two species of possum, T. vulpecula and Pseudocheirus peregrinus (Hemsley et al., 1996) and the northern brown bandicoot (Old and Deane, 2002a), descriptions of GALT in adult macropod species are limited to three species, the quokka (Ashman and
Papadimitriou, 1975), the tammar wallaby (Hemsley et al., 1995; Old and Deane, 2002b) and the Eastern grey kangaroo (Old and Deane, 2001). In the tammar wallaby, aggregations of lymphocytes were identified in the submucosa but no structured follicle arrangement such as a Peyer’s patch was described (Basden et al. 1997; Old and Deane, 2002a). In contrast, in the Eastern grey kangaroo, GALT was identified as clearly defined Peyer’s patches supported by isolated lymphocytes within the villi (Old and Deane, 2001). Given the lack of controlled studies involving the development of GALT in these species, it is difficult to determine whether the lymphoid tissue of the gut develops in response to antigenic challenge or whether it is present from birth. In this study, the presence of a Peyer’s patch in the gut of the acid-fast positive animal, Rufous Hare-wallaby 241, suggests that this concentrated lymphoid response may have arisen due to direct antigenic challenge.

**d. Bronchus Associated Lymphoid Tissue (BALT)**

In eutherian mammals, lymphocytes play an important role in the immunological protection of the lung. This is demonstrated by the numbers of interstitial lymphocytes found in human and rat lungs, where they equal those of the peripheral blood (Pabst and Tschernig, 1995). BALT, identified as small aggregations of lymphocytes lining the bronchi or situated between the bronchi and a blood vessel, was identified in all Rufous Hare-wallabies and all but one potoroo tissue in this study. The lung tissue of the pouch young of Long-footed potoroo 224 did not possess mature lung tissue and was without organised BALT. Lymphocyte infiltration of the lung parenchyma was however evident in this sample but it is difficult to assess whether these cells were pathological in nature or merely a part of this animal’s developing immune system. BALT is first observed in the lungs of young possums at 105 days of age, approximately 20 days before pouch exit (Cooke and Alley, 2002). Whilst the age of the young animal was unknown, it was still attached to the teat of the mother, which suggests that it was still a long way from maturity.

The presence of BALT within the host lung generally assumes previous antigenic contact although this is not necessarily the case in all mammalian species. In humans and pigs, BALT arises in response to antigen stimulation, whilst in rabbits and rats, germ-free animals possess these lymphoid aggregations (Pabst and Binns, 1994).
Animals investigated in this study were not from pathogen-free environments and it is thus difficult to determine if these areas of BALT arose as a consequence of direct antigenic challenge or whether they were present from birth. It is important for the nature of the present study to confirm the presence of this tissue since it implies that the respiratory system of macropods has the capacity to mount a protective immune response within the tissue.

Adult macropod lungs are structurally similar to eutherian lungs except for the presence of longer respiratory bronchioles and elongated and enlarged alveolar ducts (Makanya et al., 2001). Morphological differences in the lungs of two other marsupials, the possum and opossum, suggest that this family of animals may also have an impaired ability to clear particulate antigens after inhalation. Possum and opossum lungs have no true bronchi below the hilus of each lung and also have reduced numbers of goblet cells in the bronchial epithelium (Cooke and Alley, 2002), which may affect the efficiency of the mucociliary escalator in the clearance of inhaled particulate antigens. In *M. tuberculosis* infections, more than 90% of inhaled bacteria are expelled by this mechanism (Nardell, cited in Fenton and Vermeulen, 1996), so this is clearly an important physiological defence against air-borne pathogens. Particulate antigens such as mycobacteria are able to reach the lung via the bronchial tract, so it is possible that the lengthening of airways, together with a reduced ability to limit the entry of air-borne pathogens, may facilitate the direct entry of large numbers of inhaled mycobacteria to the alveoli. The structure of marsupial lungs may therefore play an important role in the initial contact with air-borne mycobacterial organisms and their ultimate ability to deal with excessive bacterial loads.

**Histopathology**

Lung tissue from Rufous Hare-wallaby 241 and Long-footed potoroos 880195 and 910156 contained evidence of a past acute inflammatory responses with consolidated areas evident within the lung parenchyma (Burkitt et al., 1997), particularly in the walls of blood vessels and bronchi. Multinucleated giant cells were also identified in lung tissue of Rufous Hare-wallabies 241 and 960106. These cells were not identified in any other tissue in this study but are often reported in association with tuberculosis infections (Burkitt et al., 1997; Thorel et al., 2001) and have previously been
identified in the lymph node of the Parma wallaby, *Macropus parma*, when affected by mycobacterial osteomyelitis (Mann *et al.*, 1982).

**Other Non-Lymphoid Tissues**

*a. Liver*

Liver samples were inspected for the presence of acid-fast bacteria and immunopathology associated with mycobacterial disease. The basic structure and organisation of the livers of Rufous Hare-wallabies and potoroos were similar to that described for other mammals (Banks, 1981; Gartner and Hyatt, 1994) with the exception of the pig and the mouse. Unlike the pig, but similar to humans, Rufous Hare-wallaby and potoroo livers have no interlobular septae that function to separate the hepatic lobules (Burkitt *et al.*, 1993). Also similar to humans and bandicoots (Cisternas and Armati, 1999), but different from mice (Chan *et al.*, 2001), no areas of haematopoiesis were evident in any of the adult potoroo or Rufous Hare-wallaby tissues.

**Histopathology**

The liver of the Long-nosed potoroo and Rufous Hare-wallaby were histologically similar to other mammalian species except for the pathology associated with granulomatous lesions in Rufous Hare-wallaby 241 and Long-nosed potoroo 950162, and fatty change evident in acid-fast positive Rufous Hare-wallaby 889518722. Granulomas composed of eosinophils, lymphocytes and macrophages of animal 241 appeared to arise within the centrlobular venules, which also contained numerous acid-fast organisms. The lesion within the Long-nosed potoroo liver was composed predominantly of lymphocytes. Fatty change, indicated by ‘white space’ due to lipid extraction during processing, was evident in the liver of animal 889518722 and Long-nosed potoroo 950162. Fatty change usually occurs in cases of alcohol poisoning in humans and extreme cases of liver dysfunction (Burkitt *et al.*, 1997).

*b. Adrenal Glands*

Adrenal glands from a number of Rufous Hare-wallabies were inspected for the presence of the acid-fast bacilli and evidence of disseminated disease. Similar to other mammals, these structures were surrounded by a fibrous capsule that enclosed clearly delineated outer cortex and inner medullary regions (Burkitt *et al.*, 1993).
Histopathology

The adrenal gland of Rufous Hare-wallaby 990107 appeared to contain a large tumour structure that infiltrated both the cortical and medullary regions (not shown). Large masses such as tumours are known to interfere with the production of steroid hormones such as cortisol (Burkitt et al., 1997), and increased production of this stress hormone may account for the susceptibility of this particular animal to infection with acid-fast bacteria. The adrenal cortex of this gland also contained a neutrophilic lesion (figures 6e and f). This structure was most likely an early neutrophilic granuloma, since it contained polymorphonuclear cells and cell debris in an amorphous proteinaceous background. Polymorphonuclear cells were also present amongst the cortical cells at the periphery of this structure, which indicates the relatively recent migration of neutrophils to the affected area.

c. Skin

Long-footed potoroo skin samples were inspected for the presence of acid-fast bacteria due to a history of skin nodules in some of these animals (see Table 2.3). Skin samples from Rufous Hare-wallabies and Long-nosed potoroos were not inspected in this study. Acid-fast bacteria were detected in histiocytic cells and were present in granulation tissue of Long-footed potoroo 880195. Lymphocytes were clearly involved in response to the bacterium, which was evident in the accumulation of these cells around lymphatic vessels (Figure 6.11c) and lymphocyte aggregations associated with structured granulomas (Figure 6.11e). In humans, cutaneous lesions are unusual in infections with M. avium and when apparent, are usually associated with disseminated disease (Lugo-Janer et al., 1990). Where they do occur, skin lesions may include ulcers, erythematous masses, nodules and plaques and rosacea-like lesions (Escalonilla et al., 1998). In Long-footed and Long-nosed potoroos, erythematous regions and nodules were observed in a number of animals during routine veterinary examinations prior to this study (information abstracted from autopsy records, Healesville Sanctuary Veterinary Clinic). Animal 941208 had small facial abscesses that were exuding yellow pus and animal 950162 had a history of skin lesions and episodes of uncharacterised lameness that may have been due to mycobacterial osteomyelitis. Animal 980239 was also reported to have small erythematous nodules over the lateral aspects of the hindlegs and the underside of the
tail. Long-footed potoroos 880195 and 820054 both had histories of erythematous skin lesions that were described histologically as “nodular infiltrates of lymphocytes and histiocytes”. Previous attempts to detect acid-fast organisms in some of these skin samples were unsuccessful, with the exception of animal 880195 where two organisms were detected. This lack of success with the detection of acid-fast organisms in skin lesions is characteristic of some forms of cutaneous mycobacterial disease in other mammals (Lugo-Janer et al., 1990).

**Generalised Pathology**

Other pathology such as erythrocyte congestion and areas of degeneration were apparent in some samples of lung and spleen. The histology of samples taken at autopsy can be misleading due to autolysis since erythrocyte infiltration is associated with post-mortem changes. Erythrocyte involvement was therefore assumed to be related to euthanasia in the absence of other evidence of a local immune response. Lymphocyte congestion also accompanied blood congestion in Rufous Hare-wallaby lung samples 970510, 990068 and 960106. Although these events may also be associated with post-mortem changes, lymphocyte involvement is most likely to be associated with the disease process since nonfocussed infiltration of lymphocytes correlates with disease consumption in other mammalian species (Mohan et al., 2001).

Neutrophils were present in significant numbers in a number of samples in the present study, including the subcapsular region of Rufous Hare-wallaby lymph node 960106 and a lesion in the adrenal cortex of Rufous Hare-wallaby 990107. These cells were also present within cellular infiltrates in degenerated lung samples. Neutrophils are especially active in the early stages of infection where they leave the blood and localise at the tissue injury site (Johnson et al., 1992). The presence of these cells indicates a state of early inflammation in these tissues, in contrast to the chronic inflammation that was apparent as areas of fibrosis and scar tissue within the lungs of mycobacterial affected Rufous Hare-wallabies and Long-footed potoroos.

In summary, the immunopathology of animal tissues included the formation of granulomas within the spleen, lungs, adrenal gland and liver. Multinucleated giant cells and areas of chronic inflammation were indications that in some animals, a
long-term immune response was at work. The involvement of a number of organs as well as the finding that lung and spleen samples were often congested and infiltrated with large numbers of lymphocytes, suggests that blood-borne dissemination of this bacteria causes the immune system of these animals to be overwhelmed, at least in those animals that eventually succumb to disease. The mechanisms of this response clearly need to be further explored.

*Comparative Pathology*

A wide spectrum of animals are affected to differing degrees by infection with atypical mycobacterial disease (see Luke, 1958, Thorel et al., 2001 and Section 6.1.3.1 for review). In summary, *M. avium* and *M. intracellulare* cause localised infection in most species, which principally involves the lymph nodes and may also involve cutaneous lesions in some animals e.g. the cat, dog and horse. Rare complications involving the eye are also reported in cats and horses. In two potoroos in the present study, uncharacterised ocular lesions were noted in the historical records for these animals. It is possible that these incidences were associated with undiagnosed mycobacterial infection.

Generalised disease involving the spleen, liver, gut, lungs but rarely the kidney does occur as a result of infection with *M. avium* in pigs, goats, horses, cattle, cats, dogs, sheep, non-human primates, exotic hoofed animals and marsupials. However, this degree of infection is most commonly associated with immunocompromised hosts (Bermudez and Champs, 1993) or those species that live in high densities or appear to have a particular susceptibility to mycobacterial infection e.g. cervids and marsupials (Thorel et al., 2001).

Lymph node involvement in atypical mycobacterial disease usually presents as enlarged lymph nodes that may or may not contain defined tubercles. In a number of different species such as cattle and pigs, clinical disease is often inapparent. Similar to the findings in the present study, *M. avium* lesions are usually described as diffuse granulomatous infiltrates with epithelioid cell perimeters. The numbers of these acid-fast bacteria present in tissue sections varies from low to high and appears to correlate with the strain of mycobacteria present. In most potaroo and Rufous Hare-wallaby samples, acid-fast bacteria were sequestered in phagocytic cells, with low numbers of
bacteria present in the tissues. Exceptions to this were tissue samples from animals that demonstrated the most marked pathological change i.e. Rufous Hare-wallaby 241 and Long-footed potoroos 880195 and 910156.

Other similarities between the immunopathology of the small macropod species investigated in the present study and other mammals are the incidence of cutaneous lesions similar to those found in cats and dogs (Latimer et al., 1997; Horn et al., 2000) and the incidence of spinal involvement similar to that described for horses (Hime and Jones, 1972) and other marsupials (see 6.1.3.1).

In most species, M. avium appears to enter the host via the intestine, where it is phagocytosed by macrophages, or via the skin, as an opportunistic infection that is dealt with by Langhans’ cells. In immunocompetent hosts, infection is contained and controlled by the development of local granulomas. In the present study, the presence of multinucleated giant cells in the lung and the incidence of non-caseating granulomas are similar to eutherian responses to non-tuberculous mycobacteria (Cooke et al. 1995; Burkitt et al. 1997). However, the involvement of the spleen and liver in Rufous Hare-wallaby and potoroo infections suggested haematogenous spread of the bacilli (Jackson et al., 1995) and, together with the unfocussed involvement of lymphocytes in the lung and spleen, is consistent with the findings of generalised disease in some, but not all, animals investigated in the current study.

The histopathology of Rufous Hare-wallaby and potoroo tissues described in the present study is consistent with infection with M. avium. Despite the susceptibility of marsupial species to mycobacterial disease (reviewed by Buddle and Young, 2000), the documentation of similar histological structures and the immunopathology associated with intracellular bacteria has demonstrated that at least at the tissue bed level, the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo are able to mount immune responses that are similar to those described for other mammalian species.

**6.4 Conclusion**

The peripheral blood cells of the Long-nosed potoroo, the Long-footed potoroo and the Rufous Hare-wallaby were characterised at the light microscope level and found
to be similar to metatherian and eutherian mammals studied to date. Using an immunocytochemical technique optimised on tammar wallaby cells, the peripheral blood T lymphocytes of these species were further characterised using a species cross-reactive antibody to the CD5 lymphocyte surface antigen.

Tissue sections of all three species were inspected for the presence of acid-fast bacteria, for basic lymphoid tissue architecture and general immunopathology. Acid-fast bacteria presumed to be *M. avium* were detected in representative samples of tissues obtained from the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo. Histological and immunohistological studies of these tissue sections revealed similar lymphoid structures to other metatherian and eutherian animals studied to date, with the exception of obvious immunopathology resulting from disease presumably associated with mycobacterial infection. Granulation tissue, extensive fibrotic trabeculae within the spleen, lymphocyte infiltrations in the lungs and spleen, the presence of macrophage-rich granulomas in the skin and lungs, a neutrophilic granuloma in the adrenal gland and the presence of multinucleated giant cells in the lungs are all consistent with the histopathology associated with *M. avium* affected metatherian and eutherian mammals and suggests that these animals have a functional immune response to intracellular pathogens.
CHAPTER SEVEN

Capacity to Generate an Immune Response

7.1 Introduction

In the previous chapter, the cells that comprise the immune system of the Rufous Hare-wallaby, the Long-nosed potoroo and the Long-footed potoroo were shown to be similar in morphology and distribution to those of other eutherian and metatherian mammals. With the exception of a small study of antigen-induced lymphocyte proliferation of the peripheral blood cells of the Long-footed potoroo (Phelan, 1996), there have been no functional studies documenting the capacity of these cells to respond to antigenic stimuli. In the current study, the cellular components of blood obtained opportunistically from the Rufous Hare-wallaby and both species of potoroo were investigated for their capacity to respond to test agents in vitro. Methods optimised on tammar wallaby cells were applied to whole blood and cells isolated from as little as 1.5 mL of blood. The limitations on the quantities of blood available for analyses, due mainly to animal welfare considerations, severely restricted the range of assays performed. However, despite these constraints, as many tests as possible were carried out in order to maximise the opportunity for collection of fundamental data and to provide a foundation for future immunological investigations of these endangered species. This chapter presents results obtained for the in vitro immunological responses of phagocytic and lymphoid cells to soluble and particulate antigens for the peripheral blood leukocytes of the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo.

7.2 Results

7.2.1 Isolation and Functional Capacity of Phagocytic Cells

7.2.1.1 Isolation and Culture of Monocyte-derived Adherent Cells

Macrophages were cultured from one sample of Rufous Hare-wallaby peripheral blood. PBMC were isolated over Ficoll-paque according to method 2.3.4.2.1a using Histopaque 1.083 as the density-gradient medium. Extensive washing with HBSS incorporating double strength antibiotics was necessary to remove bacteria from the blood sample that had presumably been introduced at the time of sampling and
increased during transport. Consequently, the recovery of PBMC-adherent cells was low. Nevertheless, equal volumes of tammar wallaby lymphocyte-conditioned media and fresh QBSF®-51 medium were applied to the recovered adherent cells that were incubated for a further 48 hours. Small numbers of developing macrophages were successfully cultured under these conditions (See Figure 7.1). However, cells with dendritic morphology were not apparent in these cultures. No attempt was made to culture monocytes from potoroo samples and Rufous Hare-wallaby macrophages were not further characterised due to the small numbers and volumes of peripheral blood available for analysis.

7.2.1.2 Isolation of Granulocytes
Granulocytes from potoroo and Rufous Hare-wallaby peripheral blood were isolated by Method 2.3.4.2.4a using either Ficoll-paque 1.077 or Histopaque 1.083. Up to 5% of the recovered cell population contained large mononuclear cells that were visible in preparations using the lower density gradient medium. Eosinophils were also present at up to 10% of these cell preparations. When assessed using the trypan blue exclusion technique (2.3.3.3.2), the viability of recovered cells was routinely >80%. Granulocytes were suspended in the desired medium (HBSS-, unless otherwise stated) and kept on ice or at RT (as indicated) until analyses were performed.

7.2.2 Capacity to Generate A Non-Specific Response
7.2.2.1 Polarisation and Chemotaxis of Granulocytes
Experimental conditions optimised for the polarisation and chemotactic responses of tammar wallaby granulocytes were used on isolated Rufous Hare-wallaby and potoroo cells as described in Chapter 4.

7.2.2.1.1 Polarisation Studies
Granulocytes isolated from Long-footed potoroo 941208 became polarised in the presence of both LPS (100μg/mL) and serum-opsonised zymosan (see Table 7.1). Untreated control cells maintained their smooth, spherical shape (Figure 7.2a) whilst cells treated with polarising agents possessed cytoplasmic extensions and ruffled or granulated cytoplasmic surfaces (Figure 7.2b). No Rufous Hare-wallaby samples were analysed for polarisation responses.
7.2.2.1.2 Chemotactic Responses

Granulocytes isolated from Long-footed potoroo 941208 were analysed using the chemotaxis chamber assay (Method 2.3.8.4.2) with the following modifications. The incubation time was extended to overnight (15hrs) and increased cell numbers (2.4 x 10⁶ cells/well) were used to improve the levels of detection of migrated cells with the MTT assay. As well as MTT quantitation, culture wells were also visually assessed for the relative numbers of migrating cells. This was necessary since the MTT assay only measures viable cells, and those cells that were stimulated to migrate through the filters may have undergone apoptosis and would therefore not necessarily be detected.

In this assay, granulocytes were treated with LPS at 100μg/mL and 1/10 dilutions of serum-opsonised S. aureus, serum-opsonised E. coli and serum-opsonised zymosan. E. coli treated samples were not subjected to MTT assessment. All bacterial agents caused significant (P<0.05) migration of granulocytes through the filter-inserts when compared with the control well, with more LPS-treated cells remaining viable after migration (Figure 7.3). In contrast, when culture wells were also visually inspected for the total numbers of migrating cells (living and dead) after treatment with these agents, the chemotactic ability of these agents was: E.coli /SOZ > S. aureus > LPS (100μg/mL) > control. This difference in ranked chemotactic ability to the test agents is most likely due to their varying abilities to induce apoptosis in activated granulocytes.
Figure 7.1: Inverted Phase Micrograph of Mala Macrophages. Low numbers of Mala PBMC-adherent cells were cultured in serum-free media. Note the granulated appearance of the cytoplasm, which is also typical of developing macrophages from eutherian mammals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Unpolarised</th>
<th>% Polarised</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77.4</td>
<td>22.6</td>
</tr>
<tr>
<td>LPS (100ug/mL)</td>
<td>7.3</td>
<td>92.7</td>
</tr>
<tr>
<td>SOZ (1/10 dilution)</td>
<td>3.8</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Table 7.1: Polarisation Responses of Granulocytes from the Long-footed Potoroo. Cells were treated with and without polarising agents and fixed with glutaraldehyde. Results were obtained by counting a minimum of 200 cells using inverted phase contrast microscopy.
Figure 7.2: Differential Interference Phase Contrast Micrographs of Granulocytes from the Long-footed Potoroo. Cells were fixed in 2.5% glutaraldehyde after treatment with polarising agents. Figure (a) shows the spherical morphology of untreated control cells after 30mins incubation at 37°C in humidified air containing 5% CO₂. Figure (b) is a micrograph of cells treated with 100μg/mL LPS for the same time. Cells have lost their spherical morphology and the cell membranes are ruffled. Note the cytoplasmic extrusions (arrows).
Figure 7.3: Relative Biomass of Migrating Granulocytes isolated from the Long-footed Potoroo. Isolated granulocytes from animal 941208 were placed in a modified chemotaxis chamber fitted with a 3μm filter. Test agents were placed in the bottom well of this chamber and cells were added to the top well above the filter. After overnight incubation at 37°C in humidified air containing 5% CO₂, the relative biomass of the viable cells that successfully migrated through the wells was quantified using the MTT assay. The test agents included LPS at 100μg/mL, a 1/10 dilution of serum opsonised S. aureus and a 1/10 dilution of serum-opsonised zymosan. The error bars represent the range in duplicate samples.
7.2.2.1.3 Phagocytosis

Qualitative studies of the phagocytic capacity of Long-nosed potoroo and Long-footed potoroo phagocytes were undertaken on granulocytes isolated from the peripheral blood of both species and on the adherent lung cells of a Long-footed potoroo affected by mycobacterial disease.

Adherent cells isolated from the lungs of Long-footed potoroo 910156 demonstrated poor ability to ingest 1μm latex beads in a serum-enriched environment (<3% of cells with positive ingestion), although transportation of the lung tissue on ice, together with the cell isolation process, may have affected this result. In contrast, granulocytes isolated from Long-footed and Long-nosed potoroo peripheral blood phagocyted 3μm latex beads in similar numbers to tammar wallaby cells when cultured under the same conditions. Zymason particles were also readily ingested by these cells (Figure 7.4).

![Figure 7.4: Phagocytosis of Inert and Biological Particles by Potoroo Cells.](image)

Figures (a) and (b) are brightfield micrographs of Long-footed potoroo granulocytes showing ingestion of 3μm latex beads and zymosan respectively. Figure (c) is a phase-contrast micrograph of Long-nosed potoroo granulocytes that have also ingested 3μm latex beads.
7.2.2.2 Oxidative Responses

7.2.2.2.1 NBT Responses

Cells within whole blood and isolated granulocytes were tested for the presence of superoxide anion using the NBT slide assay (Method 2.3.8.4.4). Whole blood preparations contained all adherent leukocytes, so that monocytes as well as granulocytes were assessed in these tests. Low levels of monocytes were also present in granulocyte test populations that were prepared using Ficoll-paque 1.077. In the presence of NBT, superoxide anion production was detected in monocytes (Figure 7.5i and ii) and eosinophils (Figure 7.5iii and iv) in both control and LPS-stimulated assays in cells from both potoroo species (see Table 7.2).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Number</th>
<th>NBT Response (positive +ve/negative -ve)</th>
<th>Whole Blood</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-footed potoroo</td>
<td>♀820054</td>
<td>monocytes +ve</td>
<td>ND</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>♀890063</td>
<td>ND</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>♀880195</td>
<td>-ve</td>
<td>+ve*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>♂941208</td>
<td>+ve*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-nosed potoroo</td>
<td>♀960995</td>
<td>ND</td>
<td>faint +ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>♂950162</td>
<td>monocytes +ve</td>
<td>faint +ve</td>
<td></td>
</tr>
<tr>
<td>Mala</td>
<td>♀241</td>
<td>ND</td>
<td>strong +ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>♀980202</td>
<td>ND</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>990107</td>
<td>ND</td>
<td>+ve</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2: NBT Responses of Mala and Potoroo Leukocytes. The NBT Slide Assay was performed on whole blood and isolated granulocytes from each species. Cells were treated with NBT at 2mg/mL and with and without LPS at 100µg/mL. Deposition of a blue precipitate in the cytoplasm of test cells was considered a positive test. * granulocytes and monocytes positive in this sample. ND not determined.

Whilst monocytes and eosinophils generated detectable levels of superoxide anion, initial experiments on whole blood cells and isolated granulocytes obtained from transported blood of both Long-footed and Long-nosed potoroos resulted in very low NBT responses when cells were stimulated with both PMA at 1µg/mL and LPS at
100μg/mL. This reduced response was also evident when experiments were undertaken at the Healesville Sanctuary site, immediately after isolation of granulocytes from freshly drawn blood. In both Long-footed and Long-nosed potoroos, NBT positive cells did not occur in any one sample at levels greater than 10% of the neutrophil population (Figure 7.7c and d). Most neutrophils were negative for NBT and maintained their characteristic morphology when stained with safranin (Figure 7.5v). For the small numbers of NBT-positive neutrophils, specific granules within the cells were visible and were only slightly enlarged compared with negative cells. In most cases, the characteristic polymorphonuclear structure was still apparent (Figure 7.5vi).

In contrast, the granulocytes isolated from a one-off sample of Brush-tailed bettong blood showed strong NBT responses to LPS at 100μg/mL (Figure 7.6). Less than 5% of the control cells contained deposits of formazan and appeared slightly larger than other cells within the population (Figure 7.6a). Greater than 80% of granulocytes from this species were NBT positive under the conditions of this test (Figure 7.6b).

Rufous Hare-wallaby samples also showed a vigorous response to treatment with 100μg/mL LPS as evidenced by enlarged cells and dark staining NBT regions (Figure 7.7b). The incidence of NBT positive cells within these samples varied between 65 and 85%.

Control samples for these analyses included untreated granulocytes as well as lymphocyte preparations. Cells within lymphocyte preparations stained red with the safranin counterstain and did not contain NBT formazan precipitates except for focal staining at the periphery of a small number of cells.
Figure 7.5: NBT Responses of Leukocytes from Long-nosed and Long-footed Potoroos. Whole blood and isolated granulocyte preparations were incubated with NBT (2mg/mL), with and without 100μg/mL LPS. Cells were counterstained with safranin to facilitate identification. A positive oxidative burst response was indicated by the deposit of a blue formazan precipitate formed as a result of NBT reduction by oxidative species. Figures (i) and (ii) are LPS-treated and control monocytes from the Long-footed potoroo. Figures (iii) and (iv) are eosinophils from LPS-treated and control cells from a Long-nosed potoroo and a Long-footed potoroo respectively. Figure (v) is two neutrophils from Long-footed potoroo blood that show some enlargement after treatment with LPS, but no NBT formazan deposition. Figure (vi) is a rare NBT positive neutrophil from LPS-treated Long-footed potoroo cells. Scale Bar 10μm.
Figure 7.6: NBT Response of Granulocytes of the Brush-tailed Bettong. Isolated granulocytes were incubated with NBT (2mg/mL) with and without LPS (100µg/mL). Normal, unstimulated neutrophil morphology is seen in figure (a). Two enlarged, slightly NBT-positive cells can also be seen (arrows). Figure (b) is a micrograph of LPS-treated bettong granulocytes incubated at the same time as the control cells. Note the enlarged cell area and the intense blue staining NBT-formazan precipitate formed as a result of stimulation of the oxidative burst response.
In order to quantify the degree of NBT produced by granulocytes from clinically normal potoroo and Rufous Hare-wallaby cells, granulocytes from Rufous Hare-wallaby 980085 and Long-footed potoroo 941208 were assessed for their ability to undergo a respiratory burst in response to LPS at 100μg/mL. Rufous Hare-wallaby granulocytes (4.48x10^5 cells) were treated with and without LPS at 100μg/mL and processed according to the colourimetric assay described in 2.3.8.4.4. Constitutive NBT expression in control cells was measured at 31.9 μg NBT reduced/10^5 cells which rose to 46.9 μg NBT reduced/10^5 cells with the addition of LPS. This equated to a respiratory burst index (RBI) of +1.47.

Long-footed potoroo granulocytes were treated with a number of different stimulating agents and the amount of NBT formazan deposited as a result of these treatments is summarised in Table 7.3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μg NBT/10⁵ cells</th>
<th>RBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>652.0</td>
<td>1.0</td>
</tr>
<tr>
<td>LPS (10μg/mL)</td>
<td>700.0</td>
<td>4.8</td>
</tr>
<tr>
<td>LPS (100μg/mL)</td>
<td>580</td>
<td>0.89</td>
</tr>
<tr>
<td>fMLP (10⁻⁷M)</td>
<td>509.5</td>
<td>0.78</td>
</tr>
<tr>
<td>PMA (1μg/mL)</td>
<td>449.0</td>
<td>0.69</td>
</tr>
<tr>
<td>SOZ (1/10)</td>
<td>459.0</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 7.3: Respiratory Burst Indices of Treated Potoroo Granulocytes.

Long-footed potoroo granulocytes had high base-line NBT responses and non-significant (P>0.1) responses to stimulants with the exception of treatment with LPS at 10μg/mL. This blood sample from animal 941208 was contaminated with bacteria at collection, which most likely pre-activated the respiratory burst response in cells from this animal. Despite this complicating factor, these results are included here to demonstrate the capacity for the cells of some members of the potoroo population to generate levels of superoxide anions in excess of those generated by granulocytes of the tammar wallaby.
Comparative Assays - Monotremes

During the course of this study, an opportunity arose to undertake comparative oxidative burst analyses on two samples from monotremes, the short-beaked echidna and the platypus. When assessed using the qualitative NBT test (Method 2.3.8.4.4), both monotreme species demonstrated strong NBT responses to LPS at 100µg/mL under the conditions of the test (Figure 7.7e and f). Cells required only 10mins of incubation under standard conditions to reach NBT positive levels that were comparable to responses detected in tammar wallaby cells. This reaction continued as the incubation time increased and at 30mins, NBT formazan deposition was markedly increased when compared with the wallaby control cells tested for the same time.

7.2.2.2.2 Detection of Peroxidase

Two Rufous Hare-wallaby PBMC samples were assessed for the presence of peroxidase positive cells using DAB/H₂O₂ (Method 2.3.8.4.6). Positive cells were detected in cells from animal 241 but granulocytes from 990107 were peroxidase negative under the conditions of the test. Similarly, the peroxidase enzyme was detected in total leukocyte preparations of both Long-footed potoroo and Long-nosed potoroo cells (not shown).
Figure 7.7: NBT Deposition in Granulocytes of Small Wallaby Species (a-d) and Two Monotremes (e, f). In these figures, NBT deposits appear as black precipitates. Figures a, b, c and d are the NBT+LPS treated granulocytes of the tammar wallaby, Mala, Long-nosed potoroo and Long-footed potoroo respectively. Note the comparatively diminished response in potoroo granulocytes, with only a small number of cells per field containing NBT formazan deposits (arrows). Figures (e) and (f) are the LPS-treated cells of the echidna and platypus respectively. Strong NBT responses were evident in these samples. Scale bar = 10μm.
7.2.3 Capacity to Generate a Specific Response

Whole blood and isolated PBMCs were assessed for their capacity to respond to PHA and Con A using the MTT proliferation assay and the culture conditions previously described for mitogen-driven proliferation experiments using tammar wallaby cells (see Chapter 5). Both QBSF®-51 medium and RPMI-1640 with and without serum supplementation were used for these assays.

7.2.3.1 Whole Blood Assays

Whilst some effects of mitogen treatment were apparent in whole blood samples analysed in this study, this trend was not significant (*P<0.05*) for either Con A or PHA when used at concentrations of 10μg/mL and 50μg/mL respectively (Table 7.4).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Stimulation Index‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con A</td>
</tr>
<tr>
<td>Long-footed potoroo</td>
<td>880195♀ 0.90 A</td>
</tr>
<tr>
<td>Long-nosed potoroo</td>
<td>950162♂ 0.90 A</td>
</tr>
<tr>
<td></td>
<td>980239♂ 1.05 A</td>
</tr>
<tr>
<td>Mala</td>
<td>*439♂ 1.16 B</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

‡ SD < 15% of mean SI
*1/20 dilution cultured in RPMI+10%FBS
A 10μg/mL; B 25μg/mL; C 50μg/mL

Table 7.4: Potoroo and Mala Whole Blood Mitogen-Driven Proliferation Responses.

7.2.3.2 PBMC Assays

Similar to findings for tammar wallaby cells, treatment of Rufous Hare-wallaby and potoroo PBMC with PHA at 50μg/mL caused significant (*P<0.05*) stimulation indices (Table 7.5). This response was optimal in QBSF®-51 media (see Figure 7.9).
<table>
<thead>
<tr>
<th>Animal</th>
<th>Number</th>
<th>Cell Number (cells/well)</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-footed potoroo</td>
<td>880195</td>
<td>$3.4 \times 10^5$</td>
<td>1.80</td>
</tr>
<tr>
<td>Long-nosed potoroo</td>
<td>950162</td>
<td>$1.4 \times 10^5$</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td>980239</td>
<td>$9.6 \times 10^4$</td>
<td>3.70</td>
</tr>
<tr>
<td>Mala</td>
<td>980085</td>
<td>$1.2 \times 10^5$</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Table 7.5: PHA-Driven Proliferation Responses of PBMC isolated from the Rufous Hare-wallaby, Long-nosed potoroo and Long-footed potoroo. PBMCs were cultured in QBSF®-51 serum-free media with and without 50μg/mL PHA for 48 hours at 37°C and 5% CO₂ in humidified air. Proliferation responses were measured using the MTT Assay.

Treatment of Rufous Hare-wallaby PBMC ($4.48 \times 10^5$cells/well) with Con A also generated a proliferative response in RPMI-1640 medium when supplemented with 10% FBS. Levels of Con A at both 5 and 10μg/mL yielded SIs of 1.43 ($OD_{test/control} = 0.505/0.354$) and 1.44 ($OD_{test/control} = 0.511/0.354$) respectively. Aggregations of cells in this medium for Rufous Hare-wallaby and potoroo samples (Figure 7.8) were similar in size and shape to those found in tammar wallaby and human PBMC cultures (Figure 5.3).
Figure 7.8: Culture Morphology of Long-nosed Potoroo PBMC in Different Media. In Figure (a), control cells are evenly distributed throughout the culture vessel. Figure (b) shows cells treated with 50μg/mL PHA and cultured in QBSF®-51 serum-free medium for 24 hours. Note the formation of cell aggregates. Figure (c) are PBMC cultured in RPMI + 10% FBS for 24hours. Scale Bar: 100μm.

Figure 7.9: Culture of Long-nosed Potoroo PBMCs in Different Media. PBMCs (1.9x10^5 cells/well) from animal 950162 were cultured in 200μL volumes of both QBSF®-51 serum-free media and RPMI medium supplemented with 10% FBS and treated with and without PHA at 50μg/mL. After incubation for 48 hours under standard conditions, cell proliferation was measured using the MTT Assay. Results are presented as mean OD ± SD.
7.2.4 Molecules that Regulate the Immune Response

7.2.4.1 Introduction

The nature and type of cytokines synthesised in response to immunogenic sources largely determines whether the host will effectively control an immune assault. In the case of infectious agents such as *M. avium* and *T. gondii* (Innes, 1997; Ghigo *et al.*, 2001), cytokines play a key role in the activation of phagocytic cells, recruitment of effector cells, promotion of the specific memory response by lymphocytes and production of other cytokines that influence both the differentiation, maturation and immunological responses of immune cells.

Cytokines from the Interferon, Tumour Necrosis Factor and Interleukin-1 families are amongst the most important of these molecules in determining the primary and ongoing response to antigenic assault. These molecules influence both the innate and specific arms of the immune response and were investigated here because of this dualistic function.

7.2.4.2 Type I Interferons

The Type 1 Interferons, IFN-α and β, are cytokines that are key modulatory molecules in the initial response to antigen and are released in large quantities by antigen presenting macrophages (Brinkmann *et al.*, 1993). In mammals, Type I interferon genes possess immunomodulatory characteristics that include anti-viral and anti-tumour activity, promotion of the expression of MHC Class 1 antigens and enhancement of the activity of Natural Killer Cells (Tovey *et al.*, 1996; Tovey and Maury, 1999). Amongst their many functions is the ability to promote the expression of T-cell regulatory genes that influence the nature of the cytokines that are produced in response to intracellular bacteria (Matikainen *et al.*, 1999; Farrar and Murphy, 2000). In a preliminary investigation of the role of these molecules in the marsupial immune response, a small PCR survey was performed on spleen and lung tissue from a Long-footed potoroo affected by mycobacterial disease.

In this study, cDNA was prepared from RNA isolated from the spleen and lung tissue of the acid-fast positive Long-footed potoroo 820054 (Method 2.3.9.2). Consensus primers for the Type I IFNs (Table 2.6) were used to amplify Type I IFN sequences using the Polymerase Chain Reaction and the products of this reaction were visualised
using agarose gel electrophoresis (Method 2.3.8.5). Single diffuse bands representing the amplified PCR products were visible on this screening gel (not shown). DNA was recovered from these bands (Method 2.3.8.6) and cloned into the pGem vector (Method 2.3.8.7). Sixteen separate clones were sequenced from this process (see Figure 7.10). The clones were identified as putative Type I IFN sequences based on the similarity of their sequence to known IFN genes from other species using the BLASTX program (accessed via ANGIS) (data not shown). After inspection of electrophoretograms for sequence integrity (see Appendix five for representative sample), a consensus alignment of 15 of these putative Type I IFN cDNA sequences was performed to identify clones with identical nucleotide sequences. Ten distinctly different cDNA sequences were identified and subjected to maximum parsimony phylogenetic analysis.

Comparison of the partial sequences obtained from the potoroo with equivalent regions from a representative group of eutherian mammals, the tammar wallaby and the chicken, was conducted using the PHYLIP evolutionary analysis software package (Felsenstein, 1989). A maximum parsimony tree of 100 bootstrap replicates was constructed based on the sequence alignments generated using the PILEUP program (accessed through ANGIS). The tree (rooted with the chicken sequence) was displayed using the Treeview program (Page, 1998). Results from this analysis demonstrate the grouping of the potoroo Type I IFN cDNA sequences with eutherian and putative tammar wallaby IFN-α genes, separate from an IFN-β clade that also contained a putative tammar wallaby IFN-β cDNA sequence (Figure 7.11).
Figure 7.10: 2% Agarose in TBE Gel of Long-footed potoroo Type I IFN PCR Product Clones. PCR products shown here were originally amplified from potoroo spleen tissue. Enzyme digests of IFN plasmid preparations were analysed by gel electrophoresis at 100V for 45mins and visualised using ethidium bromide fluorescence under ultraviolet (UV) light. Excised PCR inserts (see line of fragments at arrow) were expected to be between 265 and 285bp. All clones shown in this gel were subsequently sequenced and identified as putative Type I IFN sequences.
Figure 7.11: Type I IFN Nucleotide Maximum Parsimony Tree. A maximum parsimony tree of 100 bootstrap replicates was constructed from nucleotide sequence alignments generated using the PILEUP program. The tree (rooted with the chicken sequence) was displayed using the Treeview program (Page, 1998). Potoroo Type 1 IFN cDNA sequences clustered with a representative tammar wallaby IFN-α sequence and with other mammalian IFN-αs forming a separate branch from the IFN-β clade. Genbank accession numbers for these sequences are recorded in Appendix 4.
7.2.4.3 TNF-α

Using the methods previously described, cDNA was prepared from RNA isolated from the PBMCs of Long-footed potoroo 941208, acid-fast positive Long-nosed potoroo 950162 and from a pooled PBMC sample from Rufous Hare-wallabies 970500, 970520 and 980202. Consensus primers developed from sequence alignments that included that of tammar wallaby TNF-α (Table 2.6) were used to amplify partial cDNA TNF-α sequence from all three of these samples (Figure 7.12). Although attempted in this study, TNF-α was not amplified from cDNA samples derived from lung, spleen, thymus and lymph node tissues of the Long-nosed and Long-footed potoroos.

After gel electrophoresis, the cDNA amplified from PBMCs was recovered and cloned into the pGem vector (Figure 7.14) and subsequently sequenced. The partial TNF-α sequences amplified in this study were compared with known sequences from *M. eugenii* and *T. vulpecula* (Figure 7.12) over the same region of DNA. After removal of primer sequences, percentage nucleotide identities for Long-footed potoroo, Long-nosed potoroo, Rufous Hare-wallaby and brushtail possum compared with the tammar wallaby were 98, 99, 99 and 81% respectively. The high degree of identity between macropod species is not surprising, since the primer sites chosen for sequence amplification were positioned in the cDNA regions showing the highest degree of consensus across a range of different species.

7.2.4.4 IL-1β

The presence of IL-1β was investigated in PBMC, lung, spleen and lymph node samples obtained from the Long-nosed and Long-footed potoroo. Despite the use of a range of PCR experimental conditions that included changes to reagent concentrations, annealing temperature and cycle numbers and times, IL-1β cDNA was not detected in any potoroo tissue.

7.2.4.5 IL-10

Partial cDNA sequence for IL-10 was obtained from PBMC isolated from acid-fast positive Long-nosed potoroo 950162 and bronchial lymph node tissue from acid-fast Long-footed potoroo 820054 (Figures 5.25 and 7.13). Bands corresponding to the
expected size of the IL-10 PCR product were also amplified in lymph node and epididymal tumour tissue from Long-nosed potoroo 950162 (also Figure 5.25).

These partial IL-10 sequences were compared with the sequence for *M. eugenii* IL-10 reported in Chapter 5 and with the Genbank sequence for *T. vulpecula* (Figure 7.14). Percentage nucleotide identities for Long-footed potoroo, Long-nosed potoroo and brushtail possum compared with the tammar wallaby were 92, 92 and 88% respectively.

A summary of the partial cytokine cDNA sequences amplified from the macropodid species in this study is presented in Table 7.6.
Figure 7.12: Comparison of TNF-α cDNA Sequences from Macropods and T. vulpecula. Nucleotides in red are different from those of the tammar wallaby sequence. The gap in the first block was introduced to conform to eutherian sequences. A single gap was also inserted in the L. hirsutus sequence in order to optimise alignments. PL P. longipes; PT P. tridactylus; LH L. hirsutus; TV T. vulpecula; ME M. eugenii. Nucleotide sequences correspond to nucleotides 371-791 of the tammar wallaby sequence (Genbank accession number: AF055915).
Figure 7.13: Ethidium Bromide Stained 2% Agarose in TBE Gel of Eco R1 Digested Plasmid Preparations Prepared from Mala and Long-footed potoroo cDNA. Lanes 3 and 4 contained putative TNF-α clones prepared from cDNA of Mala and Long-footed potoroo (LFPot) respectively. Lane 5 contained a putative IL-10 clone prepared from Long-footed potoroo lymph node tissue. Lanes 6 and 7 were clones prepared from amplified fragments that were presumed to be IL-1β from the Long-nosed potoroo and IL-8 from the Long-footed potoroo respectively. Both of these plasmids were found to contain irrelevant sequences.
Figure 7.14: Comparison of IL-10 cDNA Sequences from Macropods and T. vulpecula.
A single gap was inserted in the P. tridactylus sequence in order to optimise alignments.
Nucleotides in red indicate differences from M. eugenii sequence. PL Potorous longipes; PT Potorous tridactylus; TV Trichosurus vulpecula; ME Macropus eugenii. Nucleotide sequences correspond to nucleotides 317-514 of the brushtail possum sequence (Genbank accession number AF026277).
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Species</th>
<th>Tammar wallaby</th>
<th>Long-footed potoroo</th>
<th>Long-nosed potoroo</th>
<th>Mala</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Harrison et al. (1999)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>NI</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>√</td>
<td>×</td>
<td>×</td>
<td>NI</td>
</tr>
<tr>
<td>Type I IFNs</td>
<td>Harrison et al. (2002)</td>
<td>√</td>
<td>NI</td>
<td>NI</td>
<td></td>
</tr>
</tbody>
</table>

✓ Partial cDNA sequence obtained in this study
× Attempted but not amplified in this study
NI Not investigated in this study

Table 7.6: Summary of Partial Cytokine cDNA Sequences Amplified from Macropod Species.
7.3 Discussion

In this study, the *in vitro* immunological responses of Rufous Hare-wallaby, Long-nosed potoroo and Long-footed potoroo peripheral blood leukocytes were found to be similar to those of the tammar wallaby and to immunocompetent eutherian mammals. One notable exception to these findings was the decreased oxidative burst responses of neutrophils from captive-bred Long-nosed and Long-footed potoroos. This reduced functionality may be related to the apparent susceptibility of these animals to mycobacterial infection, since neutrophils play an important role in the control and clearance of these opportunistic pathogens (see below). Furthermore, the detection of the immunomodulatory cytokines TNF-α and IL-10, but not IL-1β, in the tissues of these animals suggests that immunoregulation by cytokine molecules is also a feature of immune responses in these species.

**Innate Responses**

**Neutrophils**

The importance of the role of neutrophil function in the aetiology of mycobacterial disease is demonstrated by the capacity of these cells to kill mycobacterial species such as *M. avium in vitro* (Brown *et al.*, 1987; Hartmann *et al.*, 2001). Neutrophils release chemotactic factors that attract macrophages to sites containing mycobacteria (Jones *et al.*, 1990) and contain defensins within their granules that have powerful *in vitro* microbicidal action against *M. avium-M. intracellulare* (Ogata *et al.*, 1992). Murine neutrophils have also been implicated in cytokine modulated non-phagocytic immune protection in *M. tuberculosis* (Pedrosa *et al.*, 2000). In the present study, results from the polarisation, chemotactic and phagocytic studies of potoroo neutrophils suggest that these species have the capacity to recognise, migrate toward and phagocytose antigenic material. These responses were similar to those of the model macropod, the tammar wallaby, and to a range of other species (see Chapter 4 Discussion). However, the capacity to provide a microbicidal response once antigen is internalised within these cells appears to be compromised, since the respiratory burst activity of these cells was significantly reduced in the neutrophils isolated from most potoroos in this study.

NBT reduction is associated with phagocytosis-mediated reactions of neutrophils, eosinophils and monocytes (DeChatelet *et al.*, 1974; Repine *et al.*, 1979; Root and
Cohen, 1981) and in the present study, monocytes and eosinophils from Long-nosed and Long-footed potoroo samples appeared to possess functional respiratory burst activity. However, neutrophils within these same samples showed depressed oxidative burst responses and in some cases, no superoxide generation was apparent in either control or LPS-treated cells.

Less than 10% of cells from both potoroo species showed localisation of NBT within cells, which is equivalent to the level of NBT positive cells found in healthy human unstimulated neutrophils (Root and Cohen, 1981). When exposed to respiratory burst stimulating agents such as LPS and PMA, over 80% of human cells reduce NBT to NBT formazan. In contrast, deposits of NBT formazan in potoroo neutrophils were low and inconsistent when treated with LPS, a treatment agent that did however successfully trigger polarisation responses in potoroo neutrophils (7.2.2.1.1) in addition to promoting strong NBT responses in tammar wallaby cells (4.2.3.1.1). The few potoroo neutrophils that were NBT positive were generally larger in size than NBT-negative cells within the same test population, although small numbers of NBT-negative cells also appeared to possess activated morphology similar to that described from tammar wallaby cells (see Chapter 4). In marked contrast to the diminished responses of most potoroo cells and similar to human and tammar wallaby cellular responses, the majority of neutrophils within Rufous Hare-wallaby blood samples showed a vigorous NBT response to treatment with LPS.

To ensure that reduced oxidative burst responses in potoroo neutrophils were not due to experimental variables associated with transport, one series of NBT experiments was undertaken at the Healesville Sanctuary site. Results from these experiments mirrored those obtained for transported blood, thus eliminating these factors as significant variables. Tammar wallaby whole blood NBT responses were also still readily detectable up to 26 hours after blood collection (see Chapter 4), which further confirmed that defects in potoroo cells were the most likely cause of the reduced NBT responses.

Quantitative analysis of NBT generated by Rufous Hare-wallaby granulocytes was within the range reported for tammar wallaby cells using similar treatment conditions (see Chapter 4). In contrast to these results and to results obtained in the NBT slide
assays, Long-footed potoroo granulocytes from animal 941208 produced superoxide anion levels up to three times higher than the maximum response seen in tammar granulocytes when treated with a similar range of stimulating agents. Consistent with these results were the high polarised cell counts for this animal, where 23% of unstimulated control cells possessed non-spherical morphology. As previously stated, pre-activation of cells within this sample as a consequence of exposure to contaminating bacteria explains these high levels. It does not explain however, the difference in the ability of the cells of this animal to produce a very strong NBT response compared with the cells from other potoroos tested in this study. One likely explanation for this apparent anomaly is the lineage of this animal, since 941208 was born in the wild and was not offspring of colony inhabitants. Also of potential significance to this study was the history of successful spontaneous regression of granulomas in this animal, which suggests a functional immune response to mycobacterial disease.

It is possible that ‘in-breeding’ effects within the Healesville colony have reduced the capacity of potoroo neutrophils to raise a normal NBT response and hence render them more susceptible to infections with opportunistic pathogens such as M. avium (Hoffman et al., 2000). An explanation for this apparent neutrophil dysfunction is the potential presence of the genetic disorder Chronic Granulomatous Disease (CGD) within the colony. CGD is a sex-linked human disorder, transmitted by heterozygous females, where deficiencies in NADH and NADPH oxidases reduce the capacity of granulocytes to mount a normal superoxide response (Nakamura, 1974; Repine et al., 1979). Other factors such as granulomatous skin reactions, shared by mycobacterial disease, are also characteristic of this disorder. Of benefit in future studies would be investigations into the significance of in-breeding on the immunological responses of these animals, which are now possible due to the identification of microsatellite markers for this species (Luikart et al., 1997). An assessment of the presence of CGD within the colony would also be of benefit to future immunological investigations.

Other Species
During this study, a single opportunistic sample of Brush-tailed Bettong blood was also obtained from Alice Springs Desert Park. This animal was previously housed with a cage-mate that succumbed to disease caused by infection with M. avium and
was thus a candidate for investigation in this work. NBT responses of granulocytes from this animal were of interest since, like the Healesville potoroos, the bettong is also a member of the Potoroinae and analysis of this sample allowed the relationship between depressed oxidative burst responses and phylogenetic considerations to be tested. Similar to Rufous Hare-wallaby and tammar wallaby granulocytes, the cells from this animal yielded a vigorous oxidative burst response, reacting strongly to stimulation with LPS in the NBT assay. This result suggests that the depressed NBT response seen in the Healesville potoroos is peculiar to those animals and not a response shared by all members of the potoroid family.

Other incidental samples analysed in this study included granulocytes from the short-beaked echidna and the platypus. Cells from both of these species produced a faster and more intense response to LPS than in any of the macropodid species. The increased kinetics of this response may reflect phylogenetic relationships between monotremes and marsupials and should be further explored in order to investigate the relationship between this apparently increased innate immune capacity and the less sophisticated specific immune response of these animals (Diener and Ealey, 1965; Jurd, 1994).

**Macrophages**

Macrophages play a primary role in the activation of lymphocytes in the immune response to mycobacterial infection (Flesch and Kaufmann, 1990) and the study of these cells *in vitro* first requires isolation and culture techniques suitable for propagation of these cells. Using culture conditions optimised on tammar wallaby cells, a small number of cells resembling peripheral blood monocytes were successfully cultured from Rufous Hare-wallaby peripheral blood. These cells displayed the characteristic granulated appearance of developing macrophages similar to both tammar wallaby and eutherian cells (Hunt, 1987). No attempt was made to isolate monocytes from either species of potoroo in this study, but now that a culture system for these cells has been established, future studies of monocyte and macrophage function in these species can be progressed.
Peroxidase
The peroxidase enzyme was localised in both Rufous Hare-wallaby and potoroo granulocytes but was not investigated in monocytes due to low available cell numbers. Similar to the results for tammar wallaby granulocytes, there was some variability in the presence of peroxidase activity in the cells from different animals. However, differences in transport times, cell preparation times and delays in peroxidase testing were considered the likely explanation for this variability, rather than any inherent cellular defects.

In summary, Long-nosed and Long-footed potoroo samples demonstrated the capacity to undergo phagocytosis when co-cultured with 3μm latex particles and the yeast-derived material, zymosan. Rufous Hare-wallaby and Brush-tailed bettong granulocytes produced strong oxidative burst responses in the presence of LPS, but neutrophils isolated from a number of different Long-nosed and Long-footed potoroos did not produce a comparable response. With the exception of one wild-born Long-footed potoroo that demonstrated the capacity of this species to produce large quantities of respiratory burst products, the Healesville Sanctuary potoroos did not possess an oxidative burst response comparable to that of other animals from within the Macropodidae. The significance of this apparent reduced capacity is untested but may be a contributing factor in the on-going susceptibility of these animals to mycobacterial infections.

Specific Responses
The cell-mediated immune response plays a major role in host defence against mycobacterial pathogens since infections that involve many species within the Mycobacteriaceae involve little or no measurable humoral component (Collins and Campbell, 1982). Brushtail possums that carry sub-clinical infections with M. bovis rarely produce positive serological responses and antibody is generally only present in significant quantities when the disease becomes terminal (Buddle et al., 1995). Thus, measurement of the functional capacity of cells involved in cell-mediated immunity is an important facet of investigation into the immunological mechanisms available to the host for resolution of mycobacterial infections (Flesch and Kaufmann, 1990).
Whole Blood
Since much of the work assaying cell-mediated immune responses requires large cell numbers, data on protected native marsupial species, particularly those that are endangered, is difficult to accumulate. In an effort to circumvent the problem of limited available cell numbers and to improve the data obtained from small blood samples, a whole blood MTT proliferation assay was successfully developed using tammar wallaby blood (see Chapter 5). Using these conditions, stimulation indices for whole blood samples from Rufous Hare-wallaby 439 reflected those obtained for the tammar (Table 5.6), with a measurable proliferation response to Con A (Table 7.4). This animal was euthanased due to ill-health but was not a candidate for presumptive mycobacteriosis. In contrast to this positive mitogen-driven proliferation response, SIs for Con A treatment of potoroo blood samples obtained from animals that were affected by mycobacterial disease were depressed or similar to control values. SIs for treatment with PHA were higher than those for Con A, but still not significant under the condition of the test. The small numbers of samples analysed in this study make it difficult to interpret these results. However, the apparently depressed responses to Con A suggest that the whole blood assay may be a suitable test for the prompt detection of cellular immunodeficiency, since reduced responses to Con A were present in animals affected by mycobacterial disease and were not apparent in healthy tammar wallabies using the same test conditions. Larger scale studies using an increased number of animals in both healthy and diseased states are now required to confirm the suitability of this test for early detection of decreased cell-mediated immune function.

PBMC
Culture conditions established for mitogen stimulation assays of tammar wallaby PBMC were confirmed for their suitability on cells from case study animals using Long-nosed potoroo PBMC. These conditions were used to perform a small number of mitogen-driven PBMC proliferation assays on cells isolated from the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo. Proliferation responses in all three species resulted in SIs greater than 1.4, which does not suggest the presence of immunological dysfunction in lymphocytes from these species. However, generalisations regarding the immunocompetency of lymphocytes are premature without information concerning the functional capacity of lymphocytes.
within secondary lymphoid tissues such as the spleen and lymph nodes. Whilst these tissues were not available for cell culture studies, results from immunopathological investigations presented in Chapter 6 confirmed that lymphocytes within these tissues were involved in a number of local responses to antigen and therefore were likely to have similar functional properties to their eutherian cell equivalents.

In a previous study of Healesville Long-footed potoroo proliferation responses reported by Phelan (1996), lymphocyte stimulation assays (LSA) using PPD and *M. avium* as antigens were used to assess the levels of exposure to mycobacteria of both captive and wild animals. In that study, PBMC from a number of captive animals yielded positive proliferation responses to these antigens whilst cells from wild caught animals were negative to the test (see Table 2.2). It is of significance that animals that tested positive to *M. avium* in the study by Phelan (animals 820054, 880195 and 910156) were those that were most severely affected by mycobacterial disease in the present study. This suggests that LSAs using specific antigen appear to correlate to the development of later mycobacterial disease in this species and may be of use in the management of these animals. Intradermal skin testing also reported by Phelan (1996) did not prove to be diagnostic, since animals that subsequently developed mycobacterial disease did not respond to PPD at the time of initial testing.

**Molecular Studies**

Whilst the capacity to generate immune responses is essential to normal immune function, the regulation of the type, timing and degree of these responses is arguably the most important consideration when dealing with the effective clearance of antigen. In this part of the study, identification of the macrophage-derived immunoregulatory molecules TNF-α, IL-1β and IL-10, together with a survey of Type I IFNs was undertaken in PBMC or tissues of macropodid species that were affected by mycobacterial disease. TNF-α, IL-1β and IL-10 are expressed in human PBMC in response to mycobacterial antigens (Barnes *et al.*, 1992) and Type I IFNs are implicated in the control of immune responses associated with intracellular pathogens (Kontsek and Konsekova, 1997).

In the PCR survey that was performed on spleen and lung tissue from Long-footed potoroo 910156 in the present study, all Type I IFN partial sequences were tentatively
identified as IFN-α sub-types, since they were contained within the branch that included eutherian IFN-α sub-types but were separate from eutherian IFN-β sequences. The presence of this number of partial cDNA sequences was not surprising since it has been estimated that the tammar wallaby has between 10 and 12 IFN-αs (Harrison et al., 2002) and humans possess up to 18 different IFN-α subtypes (Tizard, 1995). Whilst the functional significance of this number of different IFN types is unknown, interferons play a central role in the induction of MHC molecules and the presentation of antigen in eutherian mammals, and directly affect macrophage functions such as phagocytosis and generation of the respiratory burst response. Given the potential for these molecules to perform similar roles in metatherian mammals, the presence and the potential role of these molecules in Long-footed potoroo tissues requires further investigation.

In addition to the identification of a number of Type I IFN molecules, TNF-α was also amplified from PBMC cDNAs of Long-footed potoroos and those of the Rufous Hare-wallaby and Long-nosed potoroo. However, this cytokine cDNA was not readily amplified in the lymphoid tissues of either species of potoroo. Similarly, IL-1β cDNA was not detected in any potoroo tissue. In contrast, IL-10 cDNA was readily amplified from PBMC, lymph node and epididymal tumour tissue from one Long-nosed potoroo and from a bronchial lymph node sample from a Long-footed potoroo. The presence of IL-1β and IL-10 cDNA was not investigated in Rufous Hare-wallaby tissues due to project time constraints.

Despite the use of less stringent PCR experimental conditions employed to promote the detection of TNF-α and IL-1β in a range of potoroo tissues, these cytokines were not easily detectable in the cDNA derived from the lymphoid tissues of animals that succumbed to mycobacterial disease. Consistent with these findings are studies of cytokine expression in some eutherian species, as in the case of mice, where increased levels of IL-10 expression and suppression of TNF-α occur after infection with M. avium (Bermudez and Champsi, 1993), and where increased immunity to M. bovis occurs in IL-10-deficient hosts (Murray and Young, 1999). In human studies, IL-10 is commonly found in high circulating levels in patients affected by mycobacterial disease (Hernandez-Pando et al., 1997), which may explain the relative ease with which the cDNA for this molecule was amplified from PBMC in the present study.
The suppression of TNF-α production in host species infected with mycobacteria has a potentially deleterious effect on disease resolution since one of the properties of this cytokine is to activate macrophages to inhibit the growth of, or kill, intracellular bacteria belonging to the *Mycobacterium avium* complex (MAC) (Bermudez and Young, 1992). IL-10 may nullify this effect resulting in increased bacterial growth within affected macrophages (Flesch and Kaufmann, 1990). The heavy burdens of acid-fast intracellular bacteria detected in the tissue macrophages of both potoroo species in the present study (Chapter 6) suggest that these microorganisms were not effectively controlled within host cells and support the proposition that IL-10 may have been a contributing factor in the progression of disease.

As well as its role in macrophage activation, TNF-α is also involved in chemokine regulation of the recruitment of lymphocytes and macrophages to newly forming granulomas associated with *M. avium* (Saunders and Cooper, 2000). The relative paucity of granulomas in acid-fast potoroo tissues documented in Chapter 6 of this study may also be a consequence of a lack of TNF-α activity in these tissues. In summary, the presence of the immunosuppressive cytokine IL-10, but not the macrophage-activating cytokines TNF-α and IL-1β, suggests a role for IL-10 regulated depression of TNF-α and IL-1β production in the lymphoid tissues of these animals that is most likely a consequence of infection with the intracellular pathogen, *M. avium*. Future studies using quantitative PCR techniques are necessary for this relationship to be verified.

The characterisation of immunoregulatory molecules in this study was undertaken with a view to performing future comparative responses and investigating possible underlying mechanisms causing immunosuppression in these animals. The sequence data obtained in this study can now be used to investigate the expression of these molecules in a larger range of tissues. Additionally, complete sequencing of the cDNA for these molecules will generate data that may be used to produce recombinant peptides suitable for use as adjuvants in future immunisation regimes (Heath and Playfair, 1992), a methodology that has already been demonstrated using brushtail possum recombinant TNF-α and IL-1β (Wedlock *et al.*, 1999b; Harrison and Wedlock, 2000).
7.4 Conclusion

The functional capacities of peripheral blood leukocytes of the Rufous Hare-wallaby, the Long-nosed potoroo and the Long-footed potoroo were demonstrated using *in vitro* cellular assays developed on cells from the model macropod, the tammar wallaby. Both innate and cell-mediated *in vitro* immune responses of these three species were similar to those of the tammar, with the exception of the depressed superoxide generating capacity of neutrophils from the Long-nosed and Long-footed potoroos.

The expression of macrophage-derived cytokines, TNF-α, IL-1β and IL-10 were also investigated within lymphoid tissues and peripheral blood cells of both potoroo species, and results from this preliminary study suggest that IL-10 may play a role in the suppression of both TNF-α and IL-1β within tissues affected by mycobacterial disease agents.
SECTION IV

Overall Findings, Discussion and Future Directions
CHAPTER EIGHT

Overall Findings

In general, characterisation of the cellular responses of the tammar wallaby, the Rufous Hare-wallaby, the Long-footed potoroo and the Long-nosed potoroo confirmed that the cells and tissues of the macropod immune system are similar in form and function to those of eutherian mammals.

This conclusion was based on the following key findings:

- The five main leukocyte types found in the blood of these marsupials were morphologically and functionally similar to those of other marsupials and to those of eutherian mammals studied to date.

- The capacity of these cells to generate in vitro responses to antigens was demonstrated by both phagocytic and lymphoid cell populations. Non-specific responses of monocytes and granulocytes confirmed that these cell populations were able to recognise, migrate toward, ingest and release bactericidal components in response to phagocytic stimuli.

- The capacity to generate cell-mediated responses was proven in whole blood and PBMCs isolated from small wallaby species, where polyclonal activators promoted in vitro proliferation responses similar to those obtained in assessments of eutherian immunological function.

- Where the cell-secreted products of stimulated phagocytic cell populations of these animals were investigated, monocytes and granulocytes directly released superoxide and nitric oxide in response to in vitro phagocytic stimuli. In addition, antimicrobial granule components were detected in neutrophils and eosinophils.

- A more detailed study of immunoregulatory molecules secreted by mitogen-stimulated PBMC confirmed the production of proliferative and anti-proliferative
molecules by these cells. The identities of these compounds were investigated at the gene expression level using PCR techniques. The expression of the macrophage-derived cytokines TNF-α, IL-1β and IL-10 was demonstrated in mitogen-treated tammar wallaby PBMCs. Further investigations also detected the expression of TNF-α, IL-10 and Type I IFN cDNA in the unstimulated PBMCs and/or the lymphoid tissues of the Long-footed potoroo, TNF-α and IL-10 cDNA in the PBMCs of the Long-nosed potoroo, and TNF-α cDNA in the PBMCs of the Rufous Hare-wallaby.

- The capacity to generate cellular responses in vivo was analysed in the Rufous Hare-wallaby and both species of potoroos by performing histological analyses of tissues obtained from clinically healthy animals and those affected by mycobacterial disease. The immunopathology associated with disease in these animal tissues included increased levels of white pulp and thickened trabeculae in the spleen, general degradation of spleen and lung tissues and infiltration of lymphocytes into affected areas. Evidence that an immune response was underway was visible as the sequestering of acid-fast organisms within macrophages of lymphoid tissues in addition to the formation of discrete epithelioid granulomas in a number of different organs.

Notwithstanding the general observations of immune function outlined above, this study also revealed a number of key differences in some elements of individual components of the immune system of these animals:

- The monocytes of the tammar wallaby appeared to be deficient in numbers or components of lysozyme granules, which was reflected in the low expression of the peroxidase enzyme in these cells.

- The basophils of the Rufous Hare-wallaby possessed a unique morphology, with large granules that were atypical when compared with the cells of other macropod species.
• The neutrophils of captive-born Long-footed and Long-nosed potoroos failed to generate significant levels of superoxide anions in response to endotoxin.

• A failure to contain mycobacteria within structured granulomas was apparent in a number of animals affected by mycobacterial disease.

• The immunosuppressive cytokine IL-10 was expressed in the tissues of animals affected by mycobacterial disease. In contrast, the macrophage-activating cytokines IL-1β and TNF-α were not detected in these tissues.

These factors and their potential role in immune dysfunction are discussed in the following section.

**Discussion and Future Directions**

*Monocytes, macrophages and monocyte-derived cells*

The monocyte cell lineage plays a pivotal role in the initiation, regulation and termination of immune responses to intracellular bacteria (Nathan, 1987). Despite the essential contribution of these cells to immune function in eutherian mammals, the functional capacity of these cells in marsupial species remains largely unknown.

In this study, a protocol for the isolation, culture and maintenance of monocytes and monocyte-derived adherent cells from the tammar wallaby was developed using serum-free conditions. Cells cultured in this system demonstrated phagocytic activity against lymphocytes, bacteria and latex particles, which confirmed their capacity to ingest intracellular bacteria. However, similar to the monocytes of the brushtail possum (Barbour, 1972), peripheral blood monocytes of the tammar wallaby did not appear to contain peroxidase-positive lysosomal granules. This may be of significance in the apparent susceptibility of marsupials to mycobacterial disease since, in the phagocytic cells of eutherian mammals, deficiencies in myeloperoxidase enzymes result in the delayed killing of microorganisms (Nakamura, 1974). Although peroxidase positivity was apparent in tammar wallaby neutrophils, the peroxidase-deficient monocytes of macropod marsupials may result in the successful colonisation of macrophages by large numbers of mycobacteria and explain the high burdens of
mycobacteria evident in the tissue macrophages of many tissues inspected in this study.

The culture system developed for the maturation of macrophages from blood-derived monocytes also supported the development of peripheral blood dendritic cells. This is believed to be the first report of such cells in marsupial blood and provides the opportunity for future studies defining their role in antigen presentation and processing within marsupial species. In eutherian mammals such as humans and mice, dendritic cells of the blood are able to capture and process antigen, and once mature, have an increased ability to influence the T cell cytokine response to microbial invasion (Demangel and Britton, 2000). This is evident in the ability of plasmacytoid dendritic cells of human blood, similar to those observed in tammar wallaby blood, to produce Type I IFNs in response to intracellular antigens (Pulendran et al., 2001). The availability of sequence information for Type I IFNs from macropod species, together with the ability to cultivate and sustain dendritic cells in culture, now provides the opportunity for investigation of the relationship between marsupial dendritic cells, mycobacteria and the role of interferon molecules in the initiation and control of immune responses to these pathogens.

**Granulocytes**

This is the first study to characterise the functional capacity of macropod marsupial granulocytes and, in general, results from phagocytic and antimicrobial assays confirm the previously untested presumption that the innate immune responses of marsupials are similar to those of their eutherian counterparts.

One exception to this finding was the reduced capacity of neutrophils, isolated from captive-born Long-footed and Long-nosed potoroos, to produce microbicidal superoxide anions in response to antigenic stimuli. As a first line of defence, neutrophils are essential for the reduction and removal of bacteria from the circulatory route and if their killing mechanisms were somehow deficient, these cells would deliver mycobacteria to macrophages in high numbers. Together with the potential for a decreased macrophage killing capacity due to myeloperoxidase deficiencies, these factors may contribute to the successful establishment of sufficient numbers of
mycobacteria in antigen-presenting cells to overwhelm the cell-mediated responses of the host.

The dysfunction in the neutrophils of potoroos was determined using the NBT slide assay, which is a recognised test used to diagnose human subjects afflicted with Chronic Granulomatous Disease (CGD) (Repine et al., 1979). This disorder is linked with deficiencies in phagocyte function (Patño et al., 1999) and between 70 and 80% of the pneumonia-associated infections carried by CGD patients are caused by atypical mycobacteria (Hoffman et al., 2000). The possible presence of this neutrophil disorder within the potoroo test colony should be investigated in order to identify or eliminate CGD as a factor contributing to the susceptibility of this particular group of animals to mycobacterial disease.

Lymphocyte Responses
Results from lymphocyte proliferation experiments demonstrated the capacity of macropod marsupials to respond to polyclonal activators, albeit under different culture conditions to those previously described. In general, increased numbers of cells and/or increased levels of mitogen were required to achieve similar levels of proliferation to those seen in the lymphocytes of eutherian mammals (Kristensen et al., 1982b). These variations may point to differences in the composition of cell subpopulations or their receptor molecules, rather than an absolute difference in the capacity of cells to respond to immune challenge. Similarly, it is possible that cell-surface sugar moieties that are perhaps more correctly measures of lectin-sugar interactions rather than cell-mediated immune responses may differ within therian mammals (eutherians and marsupials), which may explain some of the apparent differences in in vitro mitogen stimulation results reported to date. Support for this proposition can be found in two earlier reports of marsupial immune assessment. In the first instance, T cells from M. domestica peripheral blood failed to form rosettes with sheep erythrocytes (Infante et al., 1991) suggesting a lack of sheep RBC receptors on these cells, receptors that are commonly found on the T cells of other mammals. Secondly, lectin-binding carbohydrates routinely used to assess immune function in human and mice lymphocytes were found to be different in both number and distribution on the surface of koala lymphocytes (Wilkinson et al., 1994). Future studies that characterise the cell-signalling molecules involved in lymphocyte
stimulation responses are necessary in order to characterise the kinetics of the mitogen response, and thus facilitate a more reliable interpretation of data arising from in vitro comparative proliferation assays.

**Molecular Identification of Cytokines**

Immunoregulatory molecules play a significant role in host defence (Kelso, 1998) and their characterisation in marsupial species will allow a more complete understanding of the workings of the immune system and shed some light on the susceptibility of this group to disease agents. There are currently no available protocols that enable the direct measurement of marsupial cytokines present in biological fluids or in cell culture systems, and the screening of supernatants for the presence of bioactive compounds described in this study is a preliminary step to enable the identification of these molecules in future studies.

In eutherian cell culture systems the T-cell cycle may be promoted by the stimulation of T lymphocytes with antigens, mitogenic lectins, or T-cell specific monoclonal antibodies, but it is the presence of IL-2 that is responsible for cell replication (Cantrell and Smith, 1984). In marsupial species studied to date, this molecule has not been identified and molecular studies to elucidate the nucleotide sequence for IL-2 in the tammar wallaby and brushtail possum have been unsuccessful (reviewed by Harrison and Wedlock, 2000). However, the ability of factors within cell culture supernatants to promote the proliferation of freshly isolated tammar wallaby PBMC in the present study suggests that IL-2 or an IL-2-like growth factor was present in these preparations. Further characterisation of the bioactive factors secreted by mitogen stimulated marsupial lymphocytes is necessary so that the identity and discrete functionality of these molecules can be more fully explored. The peptide sequencing of these factors is now a possibility given that a culture system has been developed in this study that allows for large scale preparation of these molecules.

Also at the molecular level, nucleotide sequencing of the entire coding regions for the cytokines identified in the present study will allow comparisons between the immunoregulatory mechanisms of different species and provide a valuable contribution to phylogenetic studies. Attempts to sequence the complete cDNA for IL-1β and IL-10 from a lymph node of the tammar wallaby using the partial
sequences elucidated in this study are currently underway using the technique of rapid amplification of cDNA ends (RACE) described by Frohman et al. (1988).

Whilst it is important to obtain this complete sequence information, the partial sequence data obtained for TNF-α, Interferon-α, IL-1β and IL-10 is also valuable since it will facilitate the development of species-specific primers for detection of these molecules in tissue specimens. This will provide the opportunity to carry out expression studies in the tissues of healthy and diseased animals and provide more detailed information about the relationship between immunoregulatory molecules. TNF-α has a major protective function in mycobacterial infections during the aclinical phase of infection and has an on-going role in protection as the disease becomes chronic (Mohan et al., 2001). IL-10 suppresses this function (Bermudez and Champsi, 1993) and also inhibits the production of oxidative defence molecules in phagocytic cells (Ghigo et al., 2001). Together with reduced levels of TNF-α, increased levels of IL-10 are implicated in the reactivation of tuberculosis in mice (Mohan et al., 2001), and the balance of these and other cytokines such as IFN-γ and IL-2 is an important element in the survival of intracellular pathogens such as Leishmania, Toxoplasma and Mycobacteria (Sieling and Modlin, 1994; Xu et al., 1994). IFN-γ and IL-2 in addition to TNF-α are associated with a protective Type I cytokine response to mycobacterial disease whereas IL-10, IL-4 and IL-5 are associated with the progressive form of this disease. Like IL-2 and IL-4, IFN-γ remains elusive in the marsupial genome and awaits characterisation by functional studies (Harrison and Wedlock, 2000).

**Immunohistology**

Antibodies to conserved regions of T and B cell surface markers have been used for some time now to investigate the lymphoid tissues of marsupial species (Canfield and Hemsley, 2000; Old and Deane, 2002b). This body of work was further extended in the present study to include a method for labelling peripheral blood T lymphocytes in macropod species (Young and Deane, 2003) and to the use of anti-CD5 and anti-CD79b antibodies in Long-footed potoroo, Long-nosed potoroo and Rufous Hare-wallaby lymphoid tissues (Young et al., 2003). These protocols and test agents can now be used for retrospective investigations of historical samples in addition to contemporary pathological analyses. Furthermore, once a registry of normal and
atypical values is established, the blood lymphocyte labelling assay has the potential for use as a diagnostic aid to monitor changes in T lymphocyte populations in response to therapeutics or simply as a guide to general immune competence.

The development of an antibody to a peptide designed from the deduced amino acid sequence of tammar wallaby TNF-α (Harrison et al., 1999) has shown some promise for future investigations of the distribution of this cytokine in paraffin-embedded marsupial tissues (personal communication, Dr. J. Old, Macquarie University). A similar approach to the cytokine sequences for IL-10 and IL-1β will provide much needed reagents for further exploration of the localisation of these molecules in tissues and at the same time, allow direct visualisation of the cell types producing these glycoproteins. The development of enzyme-linked immunosorbant assays to enable the measurement of these molecules in biological fluids is also a future application of the antibodies to these peptides.

**Susceptibility to Mycobacterial Disease**

In eutherian mammals, susceptibility to mycobacterial disease is attributed to a number of different factors that are related to the immune system of the host as well as to the properties of the mycobacterial species in question (Saunders and Cooper, 2000). In cases of disseminated disease of apparently healthy adult humans caused by *M. avium*, specific immune defects have rarely been identified (Nedoroš et al., 1991) although genetic predisposition may play a part in susceptibility with tuberculosis mycobacteria (Chackerian et al., 2001). In mice, genetic susceptibility to mycobacteria and other intracellular pathogens has been attributed to a number of genes that include the natural resistance-associated macrophage protein gene, *Nramp1* (Gomes and Appelberg, 1998), and to other genes found within the MHC (Nedorost et al., 1991). These gene associations are still somewhat controversial though, since the presence of at least one group, the *Nramp* genes, does not correlate with disease when tested in large groups (Schaible et al., 1999). Nevertheless, genetic predisposition in marsupial species, particularly those originating from island populations, is an important factor to consider since evolutionary pressures may have influenced gene selection in these animals. Since the type of T cell cytokine response generated to a given antigen may be influenced by the degree of previous contact with a given pathogen (Kelso, 1998), the previous geographical isolation of some marsupial
species may be relevant to their ability to mount an immune response to a pathogen that was absent from these environments. This antigen naïveté may be a major factor in determining the effectiveness of the immunological responses of animals descended from island populations, where they have recently relocated to the mainland and been exposed to a milieu of previously unencountered pathogenic organisms.

There is some evidence to support the proposal that prior exposure to various types of environmental mycobacterial antigens may influence the susceptibility of host organisms to mycobacterial disease (Hernandez-Pando et al., 1997). In mice, low priming doses of M. vaccae followed by infection with M. tuberculosis generally yield a protective type 1 cytokine pattern, whereas high doses of antigens yield a mixed type 1 and type 2 cytokine response. Whether or not this type of antigen-controlled cytokine response is underway in animals such as the Long-nosed and Long-footed potoroo, it is likely that all species tested in this study are exposed to mycobacterial antigens in their local environments, particularly the potoroo species that forage for fungi within the soil (Wallis et al., 1997).

In eutherian mammals, disease arising from mycobacterial infection may be due to reactivation of a dormant infection rather than to infection with these pathogens upon initial contact (Turner et al., 2001). Thus, factors such as subclinical viral infections that compromise the immune systems of host animals may influence the course of mycobacterial disease. Similarly, the presence of corticosteroids, typically produced in stress responses (Dhabhar et al., 1995), may depress immune function and thus also contribute to the reactivation of latent bacterial or viral infections (Guliani et al., 1999). The susceptibility to mycobacterial disease of small macropod species within wild populations remains unknown and it is possible that the affects of captivity are largely responsible for the apparent sensitivity of these animals to intracellular pathogens (Jackson et al., 1995; Buddle and Young, 2000). Future studies designed to document the affects of mycobacteria on macropods within their natural environments need to be undertaken in order to clarify this issue.

Given that the causes for establishment of mycobacterial infection in eutherian species is multifactorial and is related to nutrition, stress, aging and depression of the
immune system by sub-clinical agents such as viruses (Saunders and Cooper, 2000), it is reasonable to assume that macropod species may be no more vulnerable to these pathogens than many other therian mammals. Future studies documenting the level of corticosteroids produced by both wild and captive animals should be performed in order to document base-line steroid levels against which capture stress can be quantified, since stress associated with captive conditions may act as the "triggering agent" for reactivation of sub-clinical disease.

Conclusion
The overall findings of this study suggest that the cellular immune responses of macropod marsupials are, for the most part, equivalent to their eutherian relatives. No single overriding factor was identified in this work that would account for the apparent susceptibility of these animals to intracellular pathogens, although a number of observations suggested that defects in cell populations may be a factor in the susceptibility of individual animals. However, since evidence of depressed immune function was apparent in those animals that had succumbed to infection with mycobacteria, it is probable that factors such as undefined stresses associated with captivity, rather than the inherent immunobiology of these species, is the predominant cause of the immunosuppression of these animals.

In summary, this study of cellular immune responses of a number of endangered small wallaby species within the family Macropodidae has provided fundamental data that will support on-going investigations into the mechanisms of the marsupial immune response. Furthermore, protocols developed to identify and assess cells and tissues involved in this response can now be used to accelerate the rate at which this knowledge base grows.
REFERENCES


phytomitogens of Marsupial leukocytes. Laboratory Animal Science 26, 777-780.

phytohaemagglutinin (PHA) of leukocytes from intact and thymectomized quokkas.
Australian Journal of Experimental Biology and Medical Science 50, 337-345.


post-switch isotypes in mammalian evolution; cloning of IgE, IgG and IgA from the
marsupial Monodelphis domestica. European Journal of Immunology 28, 2738-2750.

CD5 Expression Is Developmentally Regulated By T Cell Receptor (TCR) Signals


Tetrazolium Reduction in Normal Human and Chronic Granulomatous Disease

population and the ability of lymphocytes to proliferate in captive brushtail possums

Brushtail Possums, Trichosurus vulpecula, Transferred From the Wild to Captivity.

System of the Brushtail Possum, Trichosurus vulpecula. The Anatomical Record 256,
354-365.

Baker, M. L., Rosenberg, G. H., Zuccolotto, P., Harrison, G. A., Deane, E. M., Miller,
Developmental and Comparative Immunology 25, 495-507.


Farrar, J. D., Murphy, K. M. (2000). Type 1 Interferons and T helper development. *Immunology Today* 21, 484-489.


Johnson, K. A. (1999). Recovery and Discovery: where we have been and where we might go with species recovery. *Australian Mammalogy* 21, 75-86.


Wedlock, D. N., Aldwell, F. E., Buddle, B. M. (1998). Nucleotide sequence of a Marsupial interleukin-10 cDNA from the Australian brushtail possum (*Trichosurus vulpecula*). *DNA Sequence* 9, 239-244.


tuberculosis and Mycobacterium avium-Infected Macrophages. The Journal of Immunology 153, 2568-2578.


Appendix One: PHA-driven Proliferation of Tammar Wallaby PBMC. This figure was compiled from data obtained on a variety of animals across a number of different experiments and illustrates the range of stimulation indices obtained for cells cultured between 3.5x10^5 cells/well and 6.0x10^5 cells/well in QBSF®-51 Serum-free medium. Where cell numbers permitted, both 25μg/mL and 50μg/mL of PHA were applied to freshly isolated PBMC. Data shown here is from the mean of triplicate samples ±SD.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Lymphocyte source</th>
<th>Cell Number ($x10^5$ cells/well)</th>
<th>OD at 550nm* unstimulated</th>
<th>OD at 550nm* stimulated</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1958</td>
<td>Lymph node</td>
<td>7.0</td>
<td>0.326±0.070</td>
<td>0.511±0.014</td>
<td>1.56</td>
</tr>
<tr>
<td>1538</td>
<td>Lymph node</td>
<td>18.0</td>
<td>0.388±0.010</td>
<td>0.634±0.022</td>
<td>1.63</td>
</tr>
<tr>
<td>1538</td>
<td>Lymph node</td>
<td>3.5</td>
<td>0.208±0.013</td>
<td>0.152±0.009</td>
<td>0.73</td>
</tr>
<tr>
<td>1958</td>
<td>Spleen</td>
<td>4.3</td>
<td>0.359±0.013</td>
<td>0.575±0.075</td>
<td>1.60</td>
</tr>
<tr>
<td>1935</td>
<td>Spleen</td>
<td>8.0</td>
<td>0.493±0.011</td>
<td>0.685±0.029‡</td>
<td>1.39</td>
</tr>
<tr>
<td>1612</td>
<td>Blood</td>
<td>1.3</td>
<td>0.061±0.005</td>
<td>0.060±0.018</td>
<td>1.02‡</td>
</tr>
<tr>
<td>592</td>
<td>Blood</td>
<td>14.0</td>
<td>1.165±0.017</td>
<td>1.198±0.019‡</td>
<td>1.03</td>
</tr>
</tbody>
</table>

*referenced to 655nm
‡ PHA at 50µg/mL
† Control cell survival only 33%

**Appendix Two: Comparison of MTT Proliferation Results for Cells Isolated from Tammar Blood, Lymph Nodes and Spleen.** A representative range of results for low and high blood lymphocyte numbers is shown here for comparison with lymph node and spleen numbers. All cells were cultured in QBSF®-51 serum free media for 48 hours and stimulated with PHA at 25µg/mL unless otherwise indicated. Optical Density results are reported as the mean of triplicate cultures ± SD.
<table>
<thead>
<tr>
<th>Animal</th>
<th>%Identity</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>53</td>
<td>70</td>
</tr>
<tr>
<td>Human</td>
<td>51</td>
<td>73</td>
</tr>
<tr>
<td>Rabbit</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>Rat</td>
<td>51</td>
<td>69</td>
</tr>
<tr>
<td>Monkey</td>
<td>49</td>
<td>70</td>
</tr>
<tr>
<td>Horse</td>
<td>53</td>
<td>65</td>
</tr>
<tr>
<td>Mouse</td>
<td>52</td>
<td>70</td>
</tr>
<tr>
<td>Sheep</td>
<td>51</td>
<td>64</td>
</tr>
<tr>
<td>Cow</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td>Goat</td>
<td>50</td>
<td>63</td>
</tr>
<tr>
<td>Deer</td>
<td>47</td>
<td>63</td>
</tr>
<tr>
<td>Pig</td>
<td>47</td>
<td>62</td>
</tr>
</tbody>
</table>

Table A: Comparison of tammar wallaby IL-1β deduced amino acid sequence with the same region of IL-1β from other mammals.

<table>
<thead>
<tr>
<th>Animal</th>
<th>%Identity</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possum</td>
<td>83</td>
<td>95</td>
</tr>
<tr>
<td>Cat</td>
<td>69</td>
<td>86</td>
</tr>
<tr>
<td>Horse</td>
<td>65</td>
<td>83</td>
</tr>
<tr>
<td>Deer</td>
<td>65</td>
<td>83</td>
</tr>
<tr>
<td>Monkey</td>
<td>63</td>
<td>83</td>
</tr>
<tr>
<td>Cow</td>
<td>65</td>
<td>81</td>
</tr>
<tr>
<td>Human</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td>Whale</td>
<td>65</td>
<td>83</td>
</tr>
<tr>
<td>Sheep</td>
<td>65</td>
<td>80</td>
</tr>
<tr>
<td>Pig</td>
<td>62</td>
<td>83</td>
</tr>
<tr>
<td>Mouse</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Rat</td>
<td>54</td>
<td>80</td>
</tr>
<tr>
<td>Dog</td>
<td>64</td>
<td>80</td>
</tr>
</tbody>
</table>

Table B: Comparison of tammar wallaby IL-10 deduced amino acid sequence with the same region of IL-10 from other mammals.

Appendix Three: Comparison of Tammar Wallaby IL-1β and IL-10 Sequences with those of Eutherian Mammals. Partial cDNA sequences obtained for the tammar wallaby were compared with the same regions from other mammals. Information presented here was compiled from the results of a BLASTX search via ANGIS.
<table>
<thead>
<tr>
<th>Sequence Identity</th>
<th>Species and IFN sub-type</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marsupial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeGen6</td>
<td>Tammar wallaby IFN-α</td>
<td>AF522877</td>
</tr>
<tr>
<td>MeGen17</td>
<td>Tammar wallaby IFN-β</td>
<td>AF522881</td>
</tr>
<tr>
<td>PIS1 PIS2 PIS3 PIS5</td>
<td>Potoroo Spleen Sequences</td>
<td></td>
</tr>
<tr>
<td>PIFS7</td>
<td>from this study</td>
<td></td>
</tr>
<tr>
<td>PIFNL1 PIL3 PIL4 PIFNL5</td>
<td>Potoroo Lung Sequences</td>
<td></td>
</tr>
<tr>
<td>PIFNL8</td>
<td>from this study</td>
<td></td>
</tr>
<tr>
<td>Eutherian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MmA1</td>
<td>Mouse IFN-α</td>
<td>X01974</td>
</tr>
<tr>
<td>MmB</td>
<td>Mouse IFN-β</td>
<td>X14029</td>
</tr>
<tr>
<td>BtA1</td>
<td>Bovine IFN-α</td>
<td>M11001</td>
</tr>
<tr>
<td>BtW1</td>
<td>Bovine IFN-ω</td>
<td>M11002</td>
</tr>
<tr>
<td>BtB1</td>
<td>Bovine IFN-β</td>
<td>M15479</td>
</tr>
<tr>
<td>HsA1 &amp; 2</td>
<td>Human IFN-α</td>
<td>J00210 &amp; V00549</td>
</tr>
<tr>
<td>HsW1</td>
<td>Human IFN-ω</td>
<td>M11003</td>
</tr>
<tr>
<td>HsB1</td>
<td>Human IFN-β</td>
<td>M28622</td>
</tr>
<tr>
<td>OaW</td>
<td>Sheep IFN-ω</td>
<td>M73245</td>
</tr>
<tr>
<td>OaTtp1</td>
<td>Sheep Trophoblast protein</td>
<td>M73242</td>
</tr>
<tr>
<td>Bird</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GgA1</td>
<td>Chicken IFN-α</td>
<td>X92476</td>
</tr>
</tbody>
</table>

Appendix Four: List of Genbank accession numbers and potoroo sample identities for partial Type I IFN sequences.
Appendix Five: Representative Sample of Electropherogram. This electropherogram was obtained for putative Type I IFN IFN-λ and is representative of the sequencing quality obtained for cDNA products generated in this study.
Appendix Six: Publications and Conference Proceedings

Publications


Submitted Journal Articles

Harrison, G. A., **Young, L. J.,** Watson, C. M., Miska, K., Miller, R. D., Deane, E. M. A Survey of Type 1 Interferons from a Marsupial and Monotreme: Implications for
the Evolution of the Type 1 Interferon Gene Family in Mammals. *Cytokine. In review.*


**Conference Papers/Proceedings/Abstracts**


