An Investigation into the

*In Vitro and In Vivo* Function of

Murine MRP-14 (S100A9)

Josie A. R. Hobbs

March 2003

Supervisors

Dr. Nancy Hogg (Cancer Research UK)

Prof. Peter Beverley (University College London)

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at the University of London
Abstract

MRP-14 and its heterodimeric partner, MRP-8 are highly expressed in neutrophils and monocytes where they constitute 40% and 1% of the total cytosolic protein respectively. They are both low molecular weight proteins that belong to the S100 family of Ca²⁺-binding proteins.

Using the air pouch model of inflammation it has been demonstrated that recombinant murine MRP-14 (rMRP-14) is a potent chemoattractant for myeloid cells in vivo (May, 1999). While rMRP-14 did not directly cause chemotaxis or activation of myeloid cells in vitro, it caused a rapid elevation in the levels of cytokines in air pouch exudate, suggesting that rMRP-14 causes leukocyte influx via an indirect mechanism. As a control for endotoxin involvement, an air pouch experiment was performed using LPS insensitive mice. rMRP-14 did not produce an influx of cells. In addition, picogram levels of LPS, equivalent to the level of endotoxin contaminaton in rMRP-14, induced similar chemokines to rMR14 in the air pouch. Therefore it is likely the effect of rMRP-14 in vivo is due to endotoxin contamination.

Myeloid cells from MRP-14⁻/⁻ mice were characterised. MRP-8 mRNA, but not protein, was present in these cells, suggesting that the stability of MRP-8 protein is dependent on MRP-14 expression. A compensatory increase in other proteins was not detected and myeloid cell development was unaffected in MRP-14⁻/⁻ mice. MRP-14⁻/⁻ neutrophils had a reduced Ca²⁺ flux in response to suboptimal levels of MIP-2. The ability of MRP-14⁻/⁻ cells to perform chemotaxis, apoptosis or superoxide burst was unaffected. In an in vivo model of pneumonia, MRP-14⁻/⁻ mice were less able to contain the infection than MRP-14⁺/+ mice. It is proposed that MRP-14 may not be dispensible for all myeloid cell functions.

The role of MRP-8 during embryonic development was also investigated. MRP-8 was expressed by cells of both maternal and fetal origin. MRP-14⁻/⁻ mice expressed reduced levels of MRP-8 and developed normally. This is in contrast to MPR-8⁻/⁻ mice that die in utero (Passey et al., 1999a). Therefore, MRP-8 appears to play an essential function during development that is independent of MRP-14.
Acknowledgments

I would like to thank Nancy my supervisor, for all her encouragement, guidance and relentless optimism. I am also very grateful to members of the MRP club, Eileen McNeill and Meg Mathies, for sharing my frustrations and understanding my MRP-8^+ rants, and Rob for his sound mousey post doctoral advice and help with the big in vivo experiments. Also thanks to Ali McDowall, Cath Giles, Melanie Laschinger, Andrew Smith and Paula Stanley, for all their support and for making the MacLab such a friendly working environment. Past lab members that deserve a special mention are Mat “S100 trivia” Robinson, Madelon Bracke, Birgit Leitinger, and Jonathan Edgeworth for our fun neutrophil collaborations. I would also like to thank Peter Beverley for all his guidance and support over the past 3 years.

I am extremely grateful to all the staff in the Biological Resources unit at Cancer Research UK. Special thanks to Barbara and Mark who have cared for the MRP-14^- mice and taught me a lot about managing a mouse colony. Thanks to Gill, Carla and Clare for all their assistance in the mouse house.

A lot of my work would have not been possible without the excellent help of members of the support labs at Cancer Research UK. Thank you to Derek, Gary, Cathy and Ayad from the FACSlab and Dave from the 2D gel lab. On a technical note, I thank George Elia and his lab for making literally 1000s of embryo sections and performing the immunohistochemistry presented in this thesis. I would also like to thank Rosemary Jeffery for the beautiful in situ hybridisations presented in this thesis and Richard Poulson for his lessons in Photoshop and microscopy.

I would like to thank Anna, Becky, Ceri, Christian, Neil, Robin, Ryan, Sarah, Toni and co. for many happy evenings in the George! Finally, thank you to Rich for removing all “!” from this thesis and for keeping me sane over three intensive months of write up!!!
Table of contents

Abstract ..........................................................................................................................2

Acknowledgments .........................................................................................................3

Table of contents ............................................................................................................4

Table of figures ............................................................................................................14

Abbreviations ..............................................................................................................18

CHAPTER 1 ................................................................................................................21

Introduction .................................................................................................................21

1.1 The innate immune system ...................................................................................21

1.1.1 The response to infection ..............................................................................21

1.1.2 Leukocyte recruitment ..................................................................................23
    Rolling ..................................................................................................................23
    Activation ..........................................................................................................26
    Firm adhesion ...................................................................................................27
    Diapedesis ..........................................................................................................29
    Migration through the basement membrane and tissues .................................30

1.1.3 The role of neutrophils in host defense ..........................................................30

1.2 Ca\(^{2+}\) signalling and Ca\(^{2+}\) binding proteins ..................................................33

1.2.1 Ca\(^{2+}\) as a secondary messenger ...............................................................33

1.2.2 Ca\(^{2+}\) influx pathways ................................................................................35

1.2.3 Ca\(^{2+}\) storage organelles ............................................................................37

1.3 The S100 protein family .....................................................................................39

1.3.1 S100 introduction ......................................................................................39
Table of contents

1.3.2 S100 protein structure .................................................................42
1.3.3 Ca^{2+} induced conformational change ........................................42
1.3.4 Ca^{2+} binding affinities of S100 proteins in vivo .........................45
1.3.5 Zn^{2+} and Cu^{2+} binding ..........................................................45
1.3.6 Target binding by S100 proteins ....................................................46
1.3.7 Gene structure and genome localisation .......................................47
1.3.8 Expression of S100 proteins .........................................................49
1.3.9 S100 expression in disease ............................................................49
1.3.10 General functions of S100 proteins .............................................50
    Intracellular functions of S100 proteins ..........................................51
    Extracellular functions of S100 proteins ........................................51
1.3.11 Receptors ....................................................................................52
1.3.12 Insights into S100 function from mouse models .......................53
1.4 The MRP proteins .........................................................................54
1.4.1 MRP introduction .......................................................................54
1.4.2 Protein structure .........................................................................55
1.4.3 Structure of MRP-8 ......................................................................57
1.4.4 Structure of MRP-14 ....................................................................58
1.4.5 Complexes of MRP-8/14 ...............................................................59
1.4.6 Cation binding ...............................................................................60
1.4.7 Subcellular localisation .................................................................61
1.4.8 Gene structure and organisation ..................................................62
I.4.9 Comparison of human and murine MRPs .................................................. 62
I.4.10 Expression of MRP proteins ................................................................. 63
  Myeloid expression .................................................................................... 63
  Epithelial expression ................................................................................ 64
  Expression during embryonic development .............................................. 65
  Extracellular expression .......................................................................... 65
  Expression associated with disease .......................................................... 66
I.4.11 Receptors ............................................................................................ 68
I.4.12 Functions of MRP-8/14 ..................................................................... 69
  Inflammation .......................................................................................... 69
  Human MRP-14 does not activate Mac-1 .................................................. 70
  Fatty acid binding ................................................................................... 71
  NADPH oxidase activation ..................................................................... 72
  Antimicrobial ......................................................................................... 73
  Inducer of cytostasis and apoptosis ......................................................... 74
  Protection from oxidative damage .......................................................... 74
  Anticoagulant ......................................................................................... 75
  Development ......................................................................................... 75
  Inhibitor of protein kinases ..................................................................... 75
1.5 S100 proteins as chemotactic agents ...................................................... 76
1.6 Aims of this thesis ................................................................................... 78

CHAPTER 2 .................................................................................................... 79

Materials and Methods .............................................................................. 79
2.1 Materials ................................................................................................. 79
  2.1.1 Stimulants, Inhibitors and other reagents ......................................... 79
  2.1.2 Buffers/Serum ............................................................................... 80
  2.1.3 Antibodies and detection reagents for flow cytometry ................. 80
2.2 Methods ................................................................................................. 82
  2.2.1 Expression of rMRP-14 ................................................................. 82
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.2</td>
<td>Purification of rMRP-14</td>
</tr>
<tr>
<td>Nickel column</td>
<td>83</td>
</tr>
<tr>
<td>Hydroxylapatite column</td>
<td>83</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Endotoxin removal and estimation</td>
</tr>
<tr>
<td>Detergent method</td>
<td>84</td>
</tr>
<tr>
<td>KuttsuClean method</td>
<td>84</td>
</tr>
<tr>
<td>Limulus Amoebocyte Lysate (LAL) Endotoxin Assay</td>
<td>84</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Protein estimation</td>
</tr>
<tr>
<td>2.2.5</td>
<td>SDS PAGE</td>
</tr>
<tr>
<td>2.2.6</td>
<td>Two-dimensional isoelectric focusing</td>
</tr>
<tr>
<td>2.2.7</td>
<td>Western blotting</td>
</tr>
<tr>
<td>2.2.8</td>
<td>Chemokine Enzyme linked immunosorbent assays (ELISA)s</td>
</tr>
<tr>
<td>KC, MIP-2, MIP-1β and MCP-1</td>
<td>87</td>
</tr>
<tr>
<td>TNF-α</td>
<td>88</td>
</tr>
<tr>
<td>2.2.9</td>
<td>Genotyping MRP-14−/− mice</td>
</tr>
<tr>
<td>Isolation of DNA from mouse tails</td>
<td>88</td>
</tr>
<tr>
<td>Primers for polymerase chain reaction (PCR)</td>
<td>89</td>
</tr>
<tr>
<td>PCR</td>
<td>89</td>
</tr>
<tr>
<td>2.2.10</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Tris-acetate (TAE) buffer</td>
<td>90</td>
</tr>
<tr>
<td>DNA loading buffer</td>
<td>90</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>90</td>
</tr>
<tr>
<td>2.2.11</td>
<td>Cell lines</td>
</tr>
<tr>
<td>RAW 264.7 cells</td>
<td>91</td>
</tr>
<tr>
<td>WEHI 3B supernatant for myeloid cell culture</td>
<td>91</td>
</tr>
<tr>
<td>2.2.12</td>
<td>Cell preparations</td>
</tr>
<tr>
<td>Murine bone marrow leukocytes</td>
<td>91</td>
</tr>
<tr>
<td>Mature bone marrow granulocytes</td>
<td>92</td>
</tr>
<tr>
<td>Murine blood mononuclear cells</td>
<td>92</td>
</tr>
<tr>
<td>Preparation of detergent soluble leukocyte extracts</td>
<td>93</td>
</tr>
<tr>
<td>2.2.13</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Staining</td>
<td>93</td>
</tr>
<tr>
<td>Absolute cell counting</td>
<td>94</td>
</tr>
</tbody>
</table>
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell sorting</td>
<td>94</td>
</tr>
<tr>
<td>2.2.14 Immunohistochemistry</td>
<td>95</td>
</tr>
<tr>
<td>2.2.15 <em>In situ</em> hybridisation and fluorescent Y chromosome staining of decidua</td>
<td>95</td>
</tr>
<tr>
<td>Preparation of decidua</td>
<td>95</td>
</tr>
<tr>
<td>In situ hybridisation</td>
<td>96</td>
</tr>
<tr>
<td>Fluorescent Y chromosome staining</td>
<td>96</td>
</tr>
<tr>
<td>2.2.16 Measurement of intracellular $\text{Ca}^{2+}$</td>
<td>97</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>97</td>
</tr>
<tr>
<td>Indo-1-acetoxy methly ester (Indo-1-AM)</td>
<td>97</td>
</tr>
<tr>
<td>2.2.17 Chemotaxis assay</td>
<td>98</td>
</tr>
<tr>
<td>2.2.18 Measurement of superoxide production</td>
<td>98</td>
</tr>
<tr>
<td>2.2.19 Apoptosis assay</td>
<td>99</td>
</tr>
<tr>
<td>2.2.20 Air pouch model of inflammation</td>
<td>99</td>
</tr>
<tr>
<td>2.2.21 Thioglycollate-induced peritonitis</td>
<td>100</td>
</tr>
<tr>
<td>2.2.22 <em>Streptococcus pneumoniae</em>-induced pneumonia</td>
<td>100</td>
</tr>
</tbody>
</table>

## CHAPTER 3 .............................................................................................................. 102

**Effect of recombinant MRP-14 *in vitro* and *in vivo*** .............................................. 102

3.1 Introduction ....................................................................................................... 102

3.2 Results ............................................................................................................... 104

3.2.1 Expression and purification of rMRP-14 ................................................. 104

3.2.2 Purification and maturation of murine bone marrow granulocytes ...... 108

3.2.3 rMRP-14 does not directly cause chemotaxis of myeloid cells.............. 109

3.2.4 rMRP-14 does not cause myeloid cell activation ...................................... 112
### Table of contents

3.2.5 rMRP-14 induces leukocyte recruitment through an indirect mechanism ............................................................................................................................. 114

3.2.6 The effect of rMRP-14 *in vivo* can reproduced *in vitro* ......................... 116

3.2.7 Specificity of the response to rMRP-14 in the air pouch ............................ 119

3.2.8 LPS contamination can explain the effect of rMRP-14 *in vivo* ............... 122

3.3 Discussion ....................................................................................................... 124

3.3.1 Expression and purification of rMRP-14 ................................................. 124

3.3.2 rMRP-14 does not cause myeloid cell activation ..................................... 124

3.3.3 rMRP-14 causes leukocyte emigration via an indirect mechanism ....... 125

3.3.4 Macrophages are a major cell type responding to rMRP-14 *in vivo* ....... 126

3.3.5 The effect of rMRP-14 is likely to be due to endotoxin contamination.. 127

3.3.6 *In vivo* studies of LPS ............................................................................... 128

3.3.7 Could rMRP-14 be a ligand for TLR4? .................................................... 130

3.3.8 Recombinant human MRP-14 is also a potent chemoattractant *in vivo* . 131

**CHAPTER 4** .............................................................................................................. 133

Initial characterisation of MRP-14*+* mice .............................................................................................................. 133

4.1 Introduction ....................................................................................................... 133

4.2 Results ............................................................................................................... 135

4.2.1 Animal husbandry ...................................................................................... 135

4.2.2 Normal tissues and organs in MRP-14*+* mice ........................................ 137

4.2.3 Lack of MRP-14 expression in MRP-14*+* monocytes and neutrophils .. 139
| 4.2.4 Absence of MRP-8 protein but not MRP-8 mRNA in MRP-14<sup>−/−</sup> neutrophils | 141 |
| 4.2.5 Lack of compensatory changes in the levels of other proteins in MRP-14<sup>−/−</sup> myeloid cells | 143 |
| 4.2.6 Expression of MRP-8 mRNA during embryonic development in MRP-14<sup>−/−</sup> mice | 147 |
| 4.2.7 Expression of MRP-8 mRNA by maternal decidual cells | 150 |
| 4.2.8 Expression of MRP-8 mRNA by placental tissue | 153 |
| 4.2.9 Normal numbers and morphology of MRP-14<sup>−/−</sup> cells | 156 |
| 4.3 Discussion | 162 |
| 4.3.1 Animal husbandry | 162 |
| 4.3.2 Lack of MRP-8 protein in myeloid cells | 163 |
| 4.3.3 Lack of compensatory changes in the levels of other proteins in MRP-14<sup>−/−</sup> mice | 164 |
| 4.3.4 Normal myeloid cell development | 165 |
| 4.3.5 Differential regulation of MRP-8 and MRP-14 mRNA expression during embryonic development | 166 |
| 4.3.6 Is MRP-8 forming homodimers? | 166 |
| 4.3.7 MRP-8 is expressed by maternal tissue | 167 |
| 4.3.8 Nature of the maternal cell type expressing MRP-8 | 167 |
| 4.3.9 MRP-8 is expressed in the labyrinth of the placenta | 168 |
| 4.3.10 Why are MRP-8<sup>−/−</sup> mice embryonic lethal? | 169 |
| 4.3.11 What role could MRP-8 be playing during placental development? | 170 |
### 4.3.12 An additional line of MRP-14<sup>−/−</sup> mice .................................................... 171

### CHAPTER 5 .............................................................................................................. 173

**Functional characterisation of MRP-14<sup>−/−</sup> myeloid cells.** .................. 173

5.1 Introduction ....................................................................................................... 173

5.2 Results ............................................................................................................... 176

5.2.1 Source of murine myeloid cells ................................................................ 176

5.2.2 Elevated basal intracellular Ca<sup>2+</sup> levels in MRP-14<sup>−/−</sup> neutrophils....... 176

5.2.3 Role of MRP-14 in chemoattractant induced Ca<sup>2+</sup> responses .............. 177

5.2.4 Normal Ca<sup>2+</sup> release from stores and across the plasma membrane in MRP-14<sup>−/−</sup> neutrophils ......................................................................................... 180

5.2.5 Normal chemotactic response of MRP-14<sup>−/−</sup> neutrophils................. 183

5.2.6 Normal superoxide burst response of MRP-14<sup>−/−</sup> neutrophils............. 183

5.2.7 Normal apoptosis response of MRP-14<sup>−/−</sup> myeloid cells ...................... 186

5.3 Discussion ......................................................................................................... 191

5.3.1 Elevated basal intracellular Ca<sup>2+</sup> levels in MRP-14<sup>−/−</sup> neutrophils ...... 191

5.3.2 Reduced responsiveness of MRP-14<sup>−/−</sup> neutrophils to MIP-2 but not fMLP ................................................................................................................................. 192

5.3.3 Differences between fMLP and MIP-2 signalling pathways ............... 193

5.3.4 MRP-14 as a Ca<sup>2+</sup> sensor ........................................................................... 196

5.3.5 Normal chemotactic response and superoxide burst of MRP-14<sup>−/−</sup> neutrophils ........................................................................................................ 196
5.3.6 Normal spontaneous and receptor mediated apoptosis of MRP-14^{+/-} myeloid cells ....................................................................................................... 198

5.3.7 Normal apoptosis of MRP-14^{+/-} myeloid cells in response to Ca^{2+} mobilisers ............................................................................................................ 199

CHAPTER 6 .............................................................................................................. 201
Response of MRP-14^{+/-} mice to an inflammatory stimulus in vivo ..................... 201

6.1 Introduction ..................................................................................................... 201

6.2 Results ............................................................................................................... 202

6.2.1 The response of MRP-14^{+/-} mice to thioglycollate-induced peritonitis... 202

6.2.2 The response of MRP-14^{+/-} mice to Streptococcus pneumoniae infection ........................................................................................................ 202

6.3 Discussion ........................................................................................................ 206

6.3.1 Normal response of MRP-14^{+/-} mice to thioglycollate-induced peritonitis. ........................................................................................................ 206

6.3.2 MRP-14^{+/-} mice show a faster transition to systemic pneumococcal infection. ........................................................................................................ 207

6.3.3 An additional line of MRP-14^{+/-} mice ...................................................... 209

6.3.4 Other in vivo models of infection ............................................................. 209

CHAPTER 7 .............................................................................................................. 211
General Discussion and Future Directions ............................................................ 211

7.1 The effect of rMRP-14 in vivo is likely to be due to endotoxin contamination ........................................................................................................ 211

7.2 Lack of MRP-8 protein in MRP-14^{+/-} myeloid cells ................................. 212
### Table of contents

7.3 Reduced sensitivity of MRP-14⁻/⁻ neutrophils to suboptimal levels of MIP-2 ............................................................................................................................. 213

7.4 Where is the defect in the MIP-2 signalling pathway? ......................... 214

7.5 MRP-14 is not essential for many myeloid functions in vitro .................. 215

7.6 A role for MRP-8 and MRP-14 during embryonic development ............ 217

7.7 The response of MRP-14⁻/⁻ mice to an inflammatory stimulus in vivo ...... 218

7.8 Future in vivo studies to investigate the function of MRP-14 ............... 218
  Models of infection ....................................................................................... 218
  Chronic inflammation .................................................................................. 219
  Adaptive immune function ......................................................................... 220
  Epithelial cell function ............................................................................... 221

References ........................................................................................................ 222

Publications arising from this thesis ................................................................. 241
Table of figures

Figure 1.1 Leukocyte recruitment to sites of inflammation .......................................24
Figure 1.2 General signalling pathways activated by chemoattractants .....................28
Figure 1.3 Ca^{2+} signalling in non-excitabile cells .........................................................34
Figure 1.4 S100 protein structure and gene organisation .....................................................41
Figure 1.5 Crystal structures of Ca^{2+}-free and Ca^{2+}-bound S100A6 .......................44
Figure 1.6 Overlay of the structures of MRP-14, MRP-8 and MRP-6 .........................56
Figure 1.7 MRP-8/14 is localised on the vascular endothelium adjacent to migrating myeloid cells ........................................................................................................67
Figure 3.1 Nickel purification of rMRP-14 from bacterial lysate ...............................105
Figure 3.2 Hydroxypatite purification of rMRP-14 from a semi-pure preparation 107
Figure 3.3 “Mature” but not “immature” bone marrow granulocytes are responsive to fMLP ....................................................................................................................110
Figure 3.4 rMRP-14 does not directly cause leukocyte chemotaxis .........................111
Figure 3.5 rMRP-14 does not cause a calcium flux or superoxide burst in mature bone marrow granulocytes ..................................................................................113
Figure 3.6 The induction of cytokines in air pouch exudate by rMRP-14 ..................115
Figure 3.7 MIP-2 expression in air pouch tissue after rMRP-14 injection ..........117
# Table of figures

Figure 3.8 Macrophages respond to rMRP-14 *in vivo* and *in vitro* .................. 118

Figure 3.9 The effect of rMRP-14 *in vitro* may be due to endotoxin contamination .................................................................................................................. 120

Figure 3.10 LPS alone induces chemokines *in vivo* and can stimulate production of TNF-α by RAW cells ........................................................................................................ 123

Figure 4.1 Targeted inactivation of the MRP-14 gene ........................................ 136

Figure 4.2 Tissue sections of spleen from MRP-14+/− and MRP-14−/− mice .......... 138

Figure 4.3 MRP-14 protein is absent in MRP-14−/− monocytes and neutrophils .... 140

Figure 4.4 MRP-14 and MRP-8 expression in MRP-14+/+ bone marrow .......... 142

Figure 4.5 MRP-14 and MRP-8 expression in MRP-14−/− bone marrow .......... 144

Figure 4.6 Sensitivity of MRP-8 and MRP-14 detection by Western blotting ....... 145

Figure 4.7 2D gel analysis of MRP-14+/+ and MRP-14−/− bone marrow lysates .... 146

Figure 4.8 Expression of MRP-8 and MRP-14 mRNA during embryonic development in MRP-14+/+ mice at 7.5 d.p.c ................................................................. 148

Figure 4.9 Expression of MRP-8 but not MRP-14 mRNA during embryonic development in MRP-14−/− mice at 7.5 d.p.c ................................................................. 149

Figure 4.10 Fluorescent Y chromosome labelling of male embryonic tissue ...... 151

Figure 4.11 Expression of MRP-8 protein during embryonic development in MRP-14+/+ mice at 7.5 d.p.c ................................................................. 152
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.12</td>
<td>Expression of MRP-8 mRNA in the labyrinth of the placenta at 10.5 d.p.c. ................................. 154</td>
</tr>
<tr>
<td>4.13</td>
<td>Expression of MRP-8 but not MRP-14 mRNA in the placenta at 10.5 d.p.c. .................................................. 155</td>
</tr>
<tr>
<td>4.14</td>
<td>Normal leukocyte populations in MRP-14+/- mice .................................................. 157</td>
</tr>
<tr>
<td>4.15</td>
<td>Neutrophil architecture is unaltered in MRP-14+/- mice .................................................. 158</td>
</tr>
<tr>
<td>4.16</td>
<td>Monocyte architecture is unaltered in MRP-14+/- mice .................................................. 159</td>
</tr>
<tr>
<td>4.17</td>
<td>Normal adhesion receptor expression on MRP-14+/- myeloid cells .............................. 161</td>
</tr>
<tr>
<td>5.1</td>
<td>Calibration of intracellular Ca^{2+} levels ........................................................................ 178</td>
</tr>
<tr>
<td>5.2</td>
<td>Normal fMLP Ca^{2+} response of MRP-14+/- neutrophils .................................................. 179</td>
</tr>
<tr>
<td>5.3</td>
<td>Reduced MIP-2 Ca^{2+} response in MRP-14+/- neutrophils .................................................. 181</td>
</tr>
<tr>
<td>5.4</td>
<td>Normal Ca^{2+} release from stores and influx across the plasma membrane in MRP-14+/- neutrophils  .................................................. 182</td>
</tr>
<tr>
<td>5.5</td>
<td>Normal chemotactic response of MRP-14+/- neutrophils to MIP-2 .................................. 184</td>
</tr>
<tr>
<td>5.6</td>
<td>Normal superoxide burst response of MRP-14+/- neutrophils .............................................. 185</td>
</tr>
<tr>
<td>5.7</td>
<td>Measurement of neutrophil and monocyte apoptosis ...................................................... 187</td>
</tr>
<tr>
<td>5.8</td>
<td>Spontaneous and TNF-α / gliotoxin-induced apoptosis and death of bone marrow neutrophils in vitro .................................................. 188</td>
</tr>
</tbody>
</table>
Table of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9</td>
<td>Apoptosis and death of myeloid cells in response to staurosporine and Ca(^{2+}) mobilisers</td>
<td>190</td>
</tr>
<tr>
<td>6.1</td>
<td>Migration of leukocytes into the peritoneum after thioglycollate Injection</td>
<td>203</td>
</tr>
<tr>
<td>6.2</td>
<td>Time course of <em>S. pneumoniae</em> infection</td>
<td>205</td>
</tr>
<tr>
<td>7.1</td>
<td>A schematic illustration of concentration-dependent neutrophil functional responses</td>
<td>216</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar fluid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(Cholamidopropyldimethylammonio)-1-propane-sulphanate</td>
</tr>
<tr>
<td>CFAg</td>
<td>Cystic fibrosis antigen</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CP-10</td>
<td>Chemotactic protein of 10 kDa</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHR</td>
<td>Di-hydrorhodamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>d.p.c.</td>
<td>Days post coitum</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionine-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>H</td>
<td>Helix</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HMWK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilised pH gradient</td>
</tr>
<tr>
<td>JAM1</td>
<td>Junctional adhesion molecule-1</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amoebocyte lysate</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MIF</td>
<td>Migration inhibitory factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRP</td>
<td>MIF-related protein</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NIF</td>
<td>Neutrophil immobilising factor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40 or ethylphenyl-polyethylene glycol</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS-A</td>
<td>Phosphate buffered saline-A</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBM</td>
<td>Perivascular basement membrane</td>
</tr>
<tr>
<td>PdBu</td>
<td>Phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-Myristate-13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>rMRP-14</td>
<td>Recombinant MRP-14</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roël Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate</td>
</tr>
<tr>
<td>TG</td>
<td>Thioglycollate</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TKR</td>
<td>Tyrosine kinase coupled receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1.1 The innate immune system

1.1.1 The response to infection

The epithelial surfaces of the body serve as an efficient barrier to most microorganisms, however when a microorganism crosses this barrier, the innate immune system serves as the first line of defense (reviewed by Medzhitov and Janeway, 1997; Medzhitov and Janeway, 2000). The innate immune system comprises a variety of effector mechanisms, such as the complement pathway, antimicrobial peptides and phagocytic cells, which are activated immediately after infection. The innate immune system uses germline-encoded pattern recognition receptors (PRRs) to recognise a variety of conserved pathogen-associated molecular patterns (PAMPs) that are shared by broad classes of microorganisms, as well as some modified self-ligands.

PRRs are a structurally diverse set of proteins that can be grouped into three categories: secreted, signalling and endocytic (Medzhitov and Janeway, 2000). Secreted PRRs include C-reactive protein and mannose binding protein. These plasma proteins bind to microbial carbohydrates and serve to activate complement and opsonise microbes for phagocytosis (Fujita, 2002). Complement (C) is a system
of more than 30 plasma and cell-surface proteins that when activated, serves to opsonise pathogens (C3b), cause the chemotaxis and activation of leukocytes (C3a, C5a), and directly kill pathogens through the formation of the “membrane-attack complex” (Fujita, 2002).

Tissue macrophages and dendritic cells (known as antigen presenting cells, (APCs)) express endocytic and signalling PRRs. These PRRs mediate pathogen uptake and delivery into lysosomes, as well as activating signalling pathways to induce the expression of inflammatory mediators, such as TNF-α and IL-1, to regulate the inflammatory response and costimulatory molecules for the induction of adaptive immunity. APCs express a diverse array of cell surface and intracellular PRRs, including Toll-like receptors (TLRs), scavenger receptors and the mannose receptor (reviewed in (Gordon, 2002)).

In mammalian species there are at least 10 TLRs that recognise a diverse array of ligands, the majority of which are PAMPs (reviewed in (Underhill and Ozinsky, 2002)). It is thought that several microbial features can be detected simultaneously by several TLRs on an APC, to elicit a reponse that is tailored to the pathogen. Some TLRs require accessory proteins to recognise their ligand. For example gram negative bacterial lipopolysaccharide (LPS) is bound in serum by LPS-binding protein, which delivers LPS to CD14 that forms a complex with TLR4 along with another accessory protein, MD-2 (Triantafilou and Triantafilou, 2002).

Inflammatory mediators cause the rapid recruitment of leukocytes to the inflammatory site. The first leukocytes to be recruited to an inflammatory site are neutrophils and monocytes, which arrive within a few hours (Henderson et al., 2003).
1.1.2 Leukocyte recruitment

Leukocytes are recruited from the blood stream to sites of inflammation via the postcapillary venules (for a review see (Kubes, 2002; Springer, 1994)). The recruitment is a multi-step process and is summarised in Figure 1.1. Leukocyte tethering and rolling, activation, firm adhesion and dipedesis comprise the classic paradigm of leukocyte recruitment. Each stage is mediated by specific families of adhesion molecules and will be discussed individually below.

Rolling

Leukocytes initially engage in transient interactions with the endothelium that manifest as rolling due to the hemodynamic shear forces. These tethering and rolling stages are predominantly mediated by the selectins and their carbohydrate ligands (for a review see (Patel et al., 2002)). The selectins are a family of calcium-dependent, type I transmembrane glycoproteins comprising an N-terminal lectin-like ligand-binding domain, an EGF-like domain followed by a variable number of consensus repeats and a short cytoplasmic domain.

P-selectin is constitutively expressed in secretory granules ("Weibel-Palade bodies") by endothelial cells, which can be rapidly mobilised to fuse with the plasma membrane following cellular activation by inflammatory mediators such as TNF-α. E-selectin is also expressed by endothelial cells but is regulated at the level of transcription following cellular activation. L-selectin is constitutively expressed by leukocytes and is rapidly shed from the surface following cellular activation.
Leukocyte recruitment to sites of inflammation

Leukocytes are recruited from the blood stream in a multistep process. Selectins expressed by activated endothelial cells (EC) interact with leukocyte carbohydrate ligands and mediate leukocyte tethering and rolling. This brings leukocytes in close contact with chemoattractants, including chemokines, which are immobilised on the endothelial cell surface. Chemoattractant binding to seven transmembrane domain receptors activates leukocyte integrins that mediate firm adhesion. Leukocyte integrins bind to members of the immunoglobulin (Ig) family of adhesion molecules, such as ICAM-1 and VCAM-1 expressed by the endothelium. Leukocytes transmigrate through endothelial cells at intercellular junctions, mediated in part by homotypic interactions between PECAM-1 molecules. Having traversed the basement membrane, neutrophils migrate towards the inflammatory site (here represented as a site of bacterial infection) in response to gradients of locally produced chemoattractants. This migration is mediated by leukocyte integrins interacting components of the extracellular matrix.
All selectins have been shown to bind to the carbohydrate sialyl Lewis\(^x\) (sLe\(^x\)) with low affinity. A variety of glycoproteins have been demonstrated to function as selectin ligands, but for many of these ligands their role has yet to demonstrated in vivo. The best characterised selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed primarily on myeloid, lymphoid and dendritic cells (Laszik et al., 1996). Neutrophils from PSGL-1\(^{-}\) mice show attenuated rolling mediated by P-selectin but not E-selectin (Yang et al., 1999).

Studies of selectin knockout mice have provided insight into the relative importance of the family members in mediating the recruitment of leukocytes to an inflammatory site (see (Patel et al., 2002) and references therein). P-selectin knockout mice show delayed leukocyte recruitment in response to an inflammatory stimulus, demonstrating that P-selectin is important in mediating the early phase of leukocyte recruitment. P/E-selectin and P/L-selectin double knockouts have severely impaired rolling, demonstrating that P-selectin plays a key role in mediating rolling in an inflammatory situation. Leukocytes from triple selectin knockout mice have an even more severely impaired ability to roll, but demonstrate that selectin-independent mechanisms exist. For example in some models of inflammation, \(\alpha_4\) integrin has been demonstrated to mediate leukocyte rolling (Kubes, 2002). There is growing evidence that engagement of selectins triggers intracellular signalling events that are likely to induce functional changes in leukocytes and endothelial cells. However this signal is not sufficient to initiate leukocyte arrest under physiological conditions (Patel et al., 2002).
Chapter 1: Introduction

**Activation**

Locally produced chemotactic factors, such as chemokines, C5a, platelet activating factor (PAF) and formylated peptides, signal through specific G protein-coupled receptors to induce firm adhesion of rolling leukocytes.

Chemokines are a large superfamily of around 40 chemotactic cytokines of 7-10 kDa that can be grouped into four main classes (CC, CXC, CX3C and C) based on the positioning of conserved cysteine residues (Baggiolini et al., 1997). The majority of chemokines are produced as secreted molecules, and are retained on the surface of endothelial cells through low affinity interactions with glycosaminoglycans. Most chemokine receptors recognise more than one chemokine and several chemokines bind to more than one receptor. Therefore the chemokine system shows great redundancy and versatility. The expression of chemokine receptors varies among different subsets of leukocytes, ensuring that chemokines stimulate the specific recruitment of leukocyte populations. For example, murine neutrophils express CXCR2 and are recruited in response to the CXC chemokines, MIP-2 and KC, whereas monocytes express CCR2 and are recruited in response to the CCL chemokine, MCP-1 (Greaves and Schall, 2000).

Another group of chemotactic factors are the N-formyl peptides (for a recent review see ((Le et al., 2002))). N-formyl peptides are produced by bacteria and mitochondria and therefore signal infection or cellular damage to the host. Recently a variety of non-formylated peptide and lipid ligands have been shown to bind to formyl peptide receptors, such as Annexin I and lipoxin A4, suggesting that the roles of these receptors may be more complex than initially thought.
In general, leukocyte chemoattractants bind to seven transmembrane domain receptors that signal through pertussis toxin-sensitive heterotrimeric G proteins of the Gi class. Upon agonist binding, heterotrimeric Gi proteins rapidly dissociate into α and βγ subunits and activate signalling molecules and pathways that are common to most leukocyte chemoattractant receptors (Figure 1.2). One exception is CD38, which catalyses the production of cyclic ADP-ribose and appears to be an essential and specific transducer of fMLP signals in mouse neutrophils (Partida-Sanchez et al., 2001).

**Firm adhesion**

Signalling pathways downstream of chemoattractant receptors induce the rapid and transient activation of integrins to promote leukocyte adhesion (Johnston and Butcher, 2002). Integrins are heterodimeric adhesion molecules composed of non-covalently associated α and β subunits. The leukocyte integrins LFA-1 (Leukocyte function-associated antigen-1; α1β2), Mac-1 (αMβ2) and VLA-4 (Very late antigen-4; α4β1) mediate adhesion through binding to members of the immunoglobulin family, ICAM-1 (Intercellular adhesion molecule-1), ICAM-2 and VCAM-1 (Vascular cell adhesion molecule) expressed by the endothelium (reviewed by (Alon and Feigelson, 2002)). The expression of ICAM-1 and VCAM-1 is induced following endothelial cell activation and bind to leukocyte β2 integrins and VLA-4 respectively.
General chemoattractants

Chemoattractant binding to G-protein-coupled receptors (GPCR) activates G proteins, which separate into $\alpha$ and $\beta_\gamma$ subunits. The $\beta_\gamma$ subunit activates phospholipases (PLC) leading to the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$) from phosphatidylinositol 4,5-bisphosphate ($PIP_2$). $IP_3$ acts on intracellular stores to cause the release of $Ca^{2+}$ into the cytosol. One chemoattractant specific pathway is the activation of CD38 by fMLP signalling. The mechanism of CD38 activation is not clear, but presumably occurs through G-proteins. CD38 converts $NAD^+$ to cyclic ADP-ribose (cADPR) which acts on ryanodine receptors to release $Ca^{2+}$ from intracellular stores.

Figure 1.2 General signalling pathways activated by chemoattractants

This figure illustrates the general signalling pathways activated by chemoattractants. Chemoattractant binding to G-protein-coupled receptors (GPCR) activates G proteins, which separate into $\alpha$ and $\beta_\gamma$ subunits. The $\beta_\gamma$ subunit activates phospholipases (PLC) leading to the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$) from phosphatidylinositol 4,5-bisphosphate ($PIP_2$). $IP_3$ acts on intracellular stores to cause the release of $Ca^{2+}$ into the cytosol. One chemoattractant specific pathway is the activation of CD38 by fMLP signalling. The mechanism of CD38 activation is not clear, but presumably occurs through G-proteins. CD38 converts $NAD^+$ to cyclic ADP-ribose (cADPR) which acts on ryanodine receptors to release $Ca^{2+}$ from intracellular stores.
Chapter 1: Introduction

Chemokines rapidly trigger conformational changes in integrins within seconds, increasing their affinity for ligand. In addition, through a different signalling pathway, chemokines stimulate integrin lateral mobility in the plasma membrane leading to an increased avidity (Reviewed in (Hogg et al., 2002; Laudanna et al., 2002)). The contribution of affinity and avidity changes to leukocyte adhesion appears to be dependent on the density of ligand. At high ligand densities affinity changes are sufficient to cause leukocyte arrest, but at low ligand densities, avidity changes are also important (Laudanna et al., 2002).

**Diapedesis**

Gradients of chemoattractants induce adherent leukocytes to migrate across the endothelium at intercellular junctions into the tissues (for a review see (Johnson-Leger et al., 2000; Luscinskas et al., 2002)). This migration is believed to involve communication between the leukocyte and endothelium, although the intercellular signalling events are not well understood. Progress has been made in identifying the endothelial lateral junctional molecules that the leukocyte must traverse.

There are two types of endothelial cell junctions: tight junctions (TJs) that are composed of a variety of transmembrane proteins (junctional adhesion molecule-1 (JAM1), occludin, claudins), and adherens junctions (AJs) that contain VE-cadherin. AJs appear to be the main complex regulating the permeability of the endothelium and VE-cadherin is a target of inflammatory mediators such as histamine and thrombin that increase vascular permeability. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) is expressed by most leukocytes and by endothelial cells and
is concentrated at the intercellular junctions, but not with AJs or TJs. PECAM-1 can mediate cell-cell adhesion through homophilic and heterophilic interactions. Studies of PECAM\(^{-}\) mice suggest that PECAM-1 plays an important role in the transmigration of neutrophils (Duncan et al., 1999).

**Migration through the basement membrane and tissues**

Very little is known about the adhesive interactions that mediate leukocyte migration across the perivascular basement membrane (PBM). Recently a PECAM-1-dependent role for \(\alpha_6\) integrins during neutrophil transmigration has been demonstrated. Interaction of neutrophil PECAM-1 with endothelial cell PECAM-1 stimulated enhanced expression of \(\alpha_6\beta_1\) on the surface of transmigrating neutrophils (Dangerfield et al., 2002) thus aiding neutrophil migration through the PBM via interactions with laminin.

Having migrated across PBM, leukocytes move through the tissues in response to local chemotactic gradients (reviewed in (Lindbom and Werr, 2002)). Members of the integrin family, especially \(\beta_1\) integrins, play a pivotal role in this migration. Integrins mediate transient and dynamic cellular contacts with a variety of extracellular matrix proteins, particularly collagen and laminin, which serve as an anchor to enable leukocyte locomotion within tissues.

**1.1.3 The role of neutrophils in host defense**

At the site of inflammation, myeloid cells play a crucial role in host defense by phagocytosing and killing invading microorganisms. Microbial killing by neutrophils
Chapter 1: Introduction

is mediated by a combination of reactive oxygen species (ROS) and cytotoxic proteases. Phagocytosing neutrophils undergo a burst of oxygen consumption, known as the oxidative burst, caused by the assembly of an active NADPH oxidase complex. In a resting cell, the oxidase is inactive and consists of one membrane bound and four cytosolic components (Babior et al., 2002). Upon activation, the cytosolic components translocate to the membrane bound component, a $b$-type cytochrome, to form a functional multi-component electron-transfer system. The $b$-type cytochrome is largely found in the membranes of neutrophil granules, although about 5% is localised in the plasma membrane. Electrons are transferred from cytoplasmic NADPH to reduce oxygen on the opposite face of the membrane, generating superoxide. Most of the superoxide reacts to form hydrogen peroxide ($H_2O_2$) and is consumed by myeloperoxidase (MPO), a major constituent of the azurophil granules. MPO oxidises chloride ions to HOCl, a highly bactericidal chemical (Hampton et al., 1998). This oxidative burst is essential for the killing of a number of microorganisms, as shown by the predisposition to infection of individuals with chronic granulomatous disease who lack a functional NADPH oxidase (Holmes et al., 1967).

Until recently, it was widely believed that ROS directly mediated the bacterial killing by neutrophils. However, mice deficient in the granule proteases cathepsin G and elastase had normal NADPH oxidase activity but were unable to resist *Aspergillus fumigatus*, *Staphylococcus aureus* and *Candida albicans* infections (Reeves et al., 2002; Tkalcevic et al., 2000). Therefore, the NADPH oxidase is necessary but not sufficient for bacterial killing. Activation of the NADPH oxidase results in an increase in phagosomal pH. Charge compensation occurs by an influx of
K⁺ into the phagosome, which allows solubilisation of granule proteases that are bound to a matrix of anionic sulfated proteoglycan (Reeves et al., 2002). Therefore bacterial killing is proposed to occur through this liberation of granule elastase and cathepsin G into the phagosome (Reeves et al., 2002).

Many defense mechanisms employed by neutrophils are potentially harmful to host tissues. For example excessive extracellular release of proteases or reactive oxygen species can damage host tissue. Consequently, activated and potentially harmful neutrophils are removed from inflammatory sites by apoptosis in order to minimise tissue damage promote resolution of inflammation (Savill, 1997). Apoptosis causes functional down-regulation of neutrophils and the retention of proteolytic granule contents before the cells are recognised and cleared by phagocytes. Signalling for apoptosis occurs through a number of independent pathways that are initiated either from triggering events within the cell or from outside the cell (reviewed in (Strasser et al., 2000)). These pathways converge to use common machinery of cell destruction that is activated by caspases. Neutrophil apoptosis can be induced by Fas and TNF-α, and delayed by factors found at an inflammatory site, such as Granulocyte/macrophage-colony stimulating factor (GM-CSF) and LPS.

During apoptosis there is proteolysis of vital cellular constituents, chromatin condensation, internucleosomal DNA cleavage, plasma membrane blebbing and randomisation of the distribution of phosphatidyl serine between the inner and outer leaflets of the plasma membrane.
Changes in intracellular Ca\(^{2+}\) play a fundamental role in many of these key neutrophil activities (reviewed in (Pettit et al., 1997), including degranulation, phagocytosis, superoxide burst, apoptosis and signalling by many seven-transmembrane domain receptors, which will be the subject of the next section.

### 1.2 Ca\(^{2+}\) signalling and Ca\(^{2+}\) binding proteins

#### 1.2.1 Ca\(^{2+}\) as a secondary messenger

In resting cells, intracellular free Ca\(^{2+}\) levels are maintained at a very low concentration, around 100 nM, which is about 10,000 fold lower than the extracellular environment. Increases in intracellular Ca\(^{2+}\) serve as a secondary messenger in numerous signal transduction pathways, which control a wide range of cellular processes such as cell-cycle progression, secretion, adhesion and cell differentiation (reviewed in (Berridge et al., 2000)).

Several classes of integral membrane proteins serve to transport Ca\(^{2+}\) across the plasma membrane that can be grouped according to three basic transporting mechanisms: ATPases, exchangers and channels. ATPases and exchangers are the two Ca\(^{2+}\) exporting systems of the plasma membrane (Figure 1.3 A). The Na\(^+\)/Ca\(^{2+}\) exchanger is a low affinity, high capacity system, it transports three Na\(^+\) into the cell for every Ca\(^{2+}\) moved out of the cell in accordance with the transmembrane electrical potential difference. The Ca\(^{2+}\) ATPase interacts with Ca\(^{2+}\) with a high affinity, but has a low transport capacity, it transports Ca\(^{2+}\) with a 1:1 stoichiometry to ATP hydrolysis.
Figure 1.3 Ca^{2+} signalling in non-excitatory cells.

(A) In resting cells, low levels of intracellular Ca^{2+} are maintained by exchangers and ATPases that remove cytosolic Ca^{2+}. There is a Na^{+}/Ca^{2+} exchanger and a Ca^{2+} ATPase in the plasma membrane, and a Ca^{2+} ATPase in the endoplasmic reticulum.

(B) Agonist binding to either G-protein-coupled receptors (GPCR) or Tyrosine kinase coupled receptors (TKR) activates phospholipase C (PLC), leading to the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). Subsequently, plasma membrane non-voltage-activated Ca^{2+} channels open, but the exact mechanisms involved have yet to be demonstrated conclusively. Intracellular Ca^{2+} store depletion, IP_3 or DAG or an increase in intracellular Ca^{2+} have all been postulated to activate these channels.

This figure is adapted from S. W. Li et al., 2002.
Chapter 1: Introduction

The Ca^{2+} ATPase plays the most important role in maintaining the gradient of Ca^{2+} across the plasma membrane. A similar Ca^{2+} ATPase is found on the endoplasmic reticulum (ER) that transports cytosolic Ca^{2+} into the lumen of the ER and is responsible for the fine regulation of cytosolic Ca^{2+}.

1.2.2 Ca^{3+} influx pathways

Cells generate Ca^{2+} signals by using both internal and external sources of Ca^{2+}. In non-excit able cells, such as leukocytes, receptor mediated Ca^{2+} influx pathways are an important and abundant mechanism for Ca^{2+} entry (Figure 1.3 B) (Li et al., 2002). Agonist binding to G-protein-coupled receptors and tyrosine kinase-coupled receptors activates phospholipase C (PLC) β and γ isoforms respectively, which leads to the generation of inositol 1,4,5-triphosphate (IP_{3}) and diacylglycerol (DAG) from phosphatidylinositol (4,5)-bisphosphate (PIP_{2}). IP_{3} binds to receptors on the ER to induce Ca^{2+} release into the cytoplasm. Other ligand-operated Ca^{3+} channels are present on the ER, including channels that are sensitive to ryanodine which are regulated by cyclic ADP ribose. Ca^{2+} itself, on the lumenal or cytoplasmic side of the ER, is also an important regulator of Ca^{2+} release from these channels.

Following the release of Ca^{2+} from intracellular stores, Ca^{2+} enters across the plasma membrane. Ca^{2+} release from intracellular stores can stimulate Ca^{2+} influx across the plasma membrane and is known as capacitative Ca^{2+} entry (reviewed in (Berridge, 1995)). By analogy with a capacitor in an electrical circuit, the Ca^{2+} stores prevent entry when they are charged up but immediately begin to promote entry as soon as calcium is discharged, for example by physiological agonists such as IP_{3} or...
pharmacological agents, such as thapsigargin. These so called “store operated channels” appear to be an almost ubiquitous feature of cells, but neither their molecular identity nor the signal that links the empty stores to channel opening have been unambiguously identified.

Several models for the mechanism of activation of the channels exist, some propose the existence of a diffusible messenger whereas others suggest that a direct interaction between the proteins in the ER and plasma membrane is involved (Taylor, 2002). Capacitative Ca\(^{2+}\) entry is not the only Ca\(^{2+}\) entry pathway regulated by receptors that couple to PLC. There is evidence that non-capacitative Ca\(^{2+}\) entry pathways exist that can be regulated by DAG or its metabolites (Taylor, 2002). In addition, an arachidonate-regulated Ca\(^{2+}\) channel has been identified that permits Ca\(^{2+}\) entry following the receptor-mediated generation of arachidonic acid (Mignen and Shuttleworth, 2000).

The identity of any receptor-mediated Ca\(^{2+}\) influx channels in leukocytes is unknown. However, the mammalian homologues of the transient receptor potential (TRP) superfamily, originally identified in Drosophila, are the most promising candidates (Li et al., 2002). Electrophysiological and pharmacological data suggest that Ca\(^{2+}\) entry pathways differ between leukocytes (Li et al., 2002). In addition, there is evidence that multiple types of Ca\(^{2+}\) influx pathway can contribute to capacitative Ca\(^{2+}\) entry responses in neutrophils, and different stimuli, e.g. fMLP and PAF, can elicit distinct patterns of influx channel activation (Itagaki et al., 2002).

Ca\(^{2+}\) is rapidly removed from the cytoplasm to restore cytosolic Ca\(^{2+}\) to resting levels. This is mediated by ATPases and exchangers (described in section
1.2.1) and in some cell types, mitochondria can also play a role in rapidly sequestering Ca\(^{2+}\) (Berridge et al., 2000).

### 1.2.3 Ca\(^{2+}\) storage organelles

In striated muscle cells specialised organelles, the sarcoplasmic reticulum (SR) serves as an intracellular store of Ca\(^{2+}\) that can be rapidly released during cell activation. In non-muscle cells, the ER is the major intracellular Ca\(^{2+}\) store. Mature neutrophils contain very little ER, which is situated towards the centre of the cell, between the nuclear lobes. In addition, neutrophils contain a specialised organelles called calciosomes, which serve as an additional Ca\(^{2+}\) store (Krause et al., 1989). Calciosomes are vesicles that are 50 – 200 nm in diameter and contain the Ca\(^{2+}\) binding protein, calsequestrin, which is also found in the SR. They are distributed throughout the neutrophil cytosol, but are mobile and move to the periphery and accumulate at sites of phagocytosis (reviewed in (Pettit et al., 1997)). Different neutrophil agonists cause the release of Ca\(^{2+}\) from different intracellular stores, for example fMLP causes Ca\(^{2+}\) release from the central Ca\(^{2+}\) store, whereas integrin engagement and clustering is reported to cause Ca\(^{2+}\) release from peripheral stores (Pettit and Hallett, 1998).

### 1.2.3 Ca\(^{2+}\) binding proteins

Ca\(^{2+}\) binding proteins can function as buffers or sensors. Ca\(^{2+}\) buffers lower the levels cytosolic free Ca\(^{2+}\) and slow its diffusion. The rapid buffering of Ca\(^{2+}\) makes it a localised messenger for effector systems that require high concentrations of Ca\(^{2+}\) for activation. Ca\(^{2+}\) sensors undergo conformational change upon Ca\(^{2+}\) binding and
transduce the Ca^{2+} signal. The variety of Ca^{2+} binding sites employed by Ca^{2+} binding proteins is limited. Most Ca^{2+} binding proteins possess an EF hand domain, an endonexin fold or a C2 region, and the most common among these is the EF-hand (reviewed by (Ikura, 1996; Lewit-Bentley and Rety, 2000)).

The EF-hand motif consists of two perpendicularly orientated α-helices and an interhelical loop, which together form a single Ca^{2+} binding site. The Ca^{2+} binding affinities of EF-hand proteins vary substantially (K_d = 10^{-4} - 10^{-9} M), and are largely determined by the residues in the loop that coordinate the Ca^{2+} ion. In many proteins, EF-hand motifs are found in pairs, and high affinity for Ca^{2+} is often the result of cooperative binding to both sites. The change in conformation on binding Ca^{2+} varies substantially among EF-hand proteins. Some domains undergo a large conformational change on binding Ca^{2+} and function as Ca^{2+} sensors, whereas others exhibit small conformational changes and function as Ca^{2+} buffers. Therefore the EF-hand is both a very general and a very versatile moiety in calcium binding proteins.

1.2.4 Ca^{2+} sensors

Elevations in intracellular Ca^{2+} are transduced by Ca^{2+} sensor proteins that undergo conformational change on binding Ca^{2+}. The binding of Ca^{2+} typically leads to the exposure of a hydrophobic surface that allows binding to target proteins to propagate the Ca^{2+} signal. For example, calmodulin is a ubiquitously expressed Ca^{2+} sensor protein that contains two pairs of EF-hand motifs. On binding Ca^{2+}, the orientation of the EF-hands is altered, allowing calmodulin to activate a wide range of target proteins (reviewed in (Hoeflich and Ikura, 2002)). Calmodulin uses a diverse range
of activation mechanisms that include causing the release of autoinhibitory domains, enzyme active site remodelling and dimerisation of ion channels.

Ca\(^{2+}\) binding to EF-hand proteins can also directly regulate their function. For example the enzymatic activity of calcineurin and the calpain family is directly dependent on Ca\(^{2+}\). Calpains are cytosolic cysteine proteases involved in the remodelling of the cytoskeleton during signal transduction. Calpain consists of catalytic and regulatory subunits, both of which possess five EF-hand domains. The mechanism by which Ca\(^{2+}\) directly activates the protease activity of calpains has been revealed by the crystal structure of m-calpain (Hosfield et al., 1999). Ca\(^{2+}\) binding to the EF-hands induces a conformational change that is transmitted to reposition residues of the catalytic triad to form a functional active site. In addition a short “anchor” sequence at the N-terminus of the catalytic domain is released from the regulatory domain and is autolysed to irreversibly activate the enzyme.

1.3 The S100 protein family

1.3.1 S100 introduction

The S100 protein family is the largest subfamily of EF-hand proteins. In contrast to calmodulin, which is ubiquitously expressed, S100 proteins are expressed in a cell specific manner. The first members of the S100 protein family were isolated in a subcellular fraction from bovine brain, which was thought to contain nervous system specific proteins (Moore, 1965). The fraction was called S100 because the constituents were soluble in 100% saturated ammonium sulphate at neutral pH.
Subsequent studies demonstrated that this fraction contained predominantly two peptides, S100A1 and S100B. Since then, an additional 17 proteins have been assigned membership to the S100 family based on amino acid sequence homology and similar structural properties (Donato, 2001; Gribenko et al., 2001).

S100 proteins are low molecular weight acidic proteins, typically around 10 - 12 kDa that exist as homo or heterodimers in solution (with the exception of Calbindin D9k that exists as a monomer). Each subunit is composed of two EF-hand motifs flanked by hydrophobic regions at either terminus and separated by a central hinge region, (shown schematically in Figure 1.4 A). The C-terminal EF-hand is canonical, having a 12 residue loop that coordinates the Ca^{2+} ion, mainly via carboxyl groups of acidic residues that are well conserved among the S100 protein family. The N-terminal EF hand has an unconventional 14 residue loop coordinating the Ca^{2+} ion, mostly through main-chain carbonyl O atoms and has a much lower affinity for Ca^{2+} (Reviewed in (Zimmer et al., 1995)). The C-terminus and the hinge region are the most divergent regions between S100 family members, suggesting that these regions may specify different roles for individual S100 proteins.

Diverse putative functions have been assigned to S100 proteins, including regulation of phosphorylation, enzymatic activity, calcium homeostasis and cytoskeletal dynamics (see section 1.3.11). In addition a number of extracellular functions have been assigned to S100 family members. However S100 proteins do not possess a signal sequence and the mechanism of their release is unknown.
Figure 1.4 S100 protein structure and gene organisation
(A) Diagram of a typical S100 protein. The \( \alpha \) helices are represented by boxes and the areas of little or no secondary structure are represented by lines. \( \text{Ca}^{2+} \) ions are represented by yellow circles. H, Helix; L, Loop.
(B), (C) Diagram of a typical S100 mRNA and gene. Blue and white boxes represent translated and untranslated sequences respectively. The numbers denote the three exons. The second exon encodes the N-terminal non-classical EF-hand and the third exon encodes the C-terminal classical EF-hand.
1.3.2 S100 protein structure

The structure of several S100 proteins has been solved using nuclear magnetic resonance (NMR), X-ray crystallography or multiple anomalous wavelength dispersion. As predicted, the structure of S100 proteins is comprised of four α-helices that form a pair of EF-hand motifs. S100 protein monomers are arranged in an anti-parallel orientation, held together by non-covalent interactions mediated through hydrophobic amino acid sidechain interactions between helices (H)1 and H4 of each monomer. The spatial arrangements of the four α-helices are maintained through hydrophobic interactions between residues in the interior of the molecule. These hydrophobic residues are highly conserved among the S100 protein family.

1.3.3 Ca^{2+} induced conformational change

In general S100 proteins bind four Ca^{2+} molecules per dimer (Heizmann and Cox, 1998). In contrast with calmodulin or troponin C that have nearly symmetric Ca^{2+} binding loops, S100 protein Ca^{2+} binding loops are markedly different. Most of the biochemical data for S100 proteins is consistent with a two step Ca^{2+} binding model, but it remains unclear which of the two sites binds Ca^{2+} first (reviewed in (Heizmann and Cox, 1998)). Comparison of the structures of S100B and S100A6 homodimers in the Ca^{2+}-free and Ca^{2+}-bound forms reveal a Ca^{2+} induced conformational switch (Drohat et al., 1996; Drohat et al., 1998; Otterbein et al., 2002). This provides strong evidence for the role of S100 proteins as Ca^{2+} sensors. The structure of human S100A6 in the Ca^{2+}-free and Ca^{2+}-bound states was solved at very high resolution.
Chapter 1: Introduction

(1.15 Å and 1.44 Å respectively) (Figure 1.5) (Otterbein et al., 2002). Ca\(^{2+}\) binding to the dimer causes a dramatic change in the global shape and charge distribution of the dimer and leads to the exposure of two symmetrically positioned hydrophobic target binding sites. The C-terminal EF-hand undergoes a major conformational change on binding Ca\(^{2+}\). H3 undergoes an 86° reorientation and leads to a repositioning of the hinge region. In addition, H4 is approximately two turns longer in the Ca\(^{2+}\)-bound state. In contrast, the positioning of H2 is only slightly altered on binding Ca\(^{2+}\) and the conformation of the N-terminal EF-hand is largely unchanged. The surface area of the S100A6 dimer increases on binding Ca\(^{2+}\). The majority of the newly exposed surface area is hydrophobic in nature and forms a hydrophobic cleft in each monomer, defined by residues in the hinge region, H3 and H4 and the C-terminal region. This hydrophobic cleft can then provide a surface for interaction with target proteins. By this mechanism, S100 dimers can crosslink two target proteins. Within an S100 heterodimer, such as MRP-8/14, differences in the monomer target binding sites will allow heterologous target proteins to be bridged to allow heterodimer-specific functions.
Figure 1.5 Crystal structures of Ca^{2+}-free and Ca^{2+}-bound S100A6
Ribbon diagrams are followed by electrostatic surface representations. Red and blue areas indicate negatively and positively charged regions, respectively. Three views of the molecules are shown for each state rotated in increments of 90°. Ca^{2+} binding brings about a large change in shape of the dimer and increases its hydrophobicity, revealing two symmetrically positioned target binding sites. Target binding sites are only indicated for one monomer. Arrows indicate the position of the Annexin and p53 binding sites based on the structures of S100A10-annexin II/S100A11-annexin I and S100B-p53. H, Helix; L, Loop; C, C-terminus.
This figure is reproduced from Otterbein et al., 2002.
1.3.4 Ca\textsuperscript{2+} binding affinities of S100 proteins in vivo

The level of intracellular Ca\textsuperscript{2+} in resting cells is 100 nM, which rises to roughly 1 μM upon cellular activation. Most S100 proteins have a low affinity for Ca\textsuperscript{2+}, binding in the μM to mM range (Heizmann and Cox, 1998). This implies that most S100 proteins would not be able to respond to the Ca\textsuperscript{2+} signals. However, the biophysical data obtained in vitro may not accurately reflect the situation in vivo. The association of S100 proteins with certain binding partners, target proteins or Zn\textsuperscript{2+} (see section 1.3.5) may increase their affinity of for Ca\textsuperscript{2+} in vivo.

1.3.5 Zn\textsuperscript{2+} and Cu\textsuperscript{2+} binding

Zn\textsuperscript{2+} binds to most of the S100 proteins at a site that is distinct from the Ca\textsuperscript{2+} binding site. Furthermore, the binding of Zn\textsuperscript{2+} can increase or decrease the affinity of some S100 proteins for Ca\textsuperscript{2+} (Heizmann and Cox, 1998). The binding of Zn\textsuperscript{2+} to S100 proteins may cause conformational change and influence their function. However the intracellular concentration of Zn\textsuperscript{2+} is exceedingly low (< 0.1 nM), and little is known about the physiological role of intracellular Zn\textsuperscript{2+}, except for its structural role in transcription factors and its catalytic role in enzymes.

Sequence analysis reveals that several S100 protein family members contain a number of histidine and cysteine residues capable of chelating Zn\textsuperscript{2+}. The HXXXH sequence is found at the C-terminus of MRP-8, MRP-14 and S100A12 (Clohessy and Golden, 1996). When present in an α-helix, this motif can bind Zn\textsuperscript{2+} with high affinity, as found in a number of naturally occuring proteins (Higaki et al., 1992).
Chapter 1: Introduction

Such a consensus sequence not found in all Zn$^{2+}$ binding S100 proteins, although all S100 proteins contain histidine and cysteine residues that are capable of chelating Zn$^{2+}$. The solved 3D structures for some family members reveal that these residues are clustered and are predicted to form Zn$^{2+}$ binding sites (Heizmann and Cox, 1998).

S100B and S100A5 (Nishikawa et al., 1997; Schafer et al., 2000) have been shown to bind Cu$^{2+}$ ions. Most Cu$^{2+}$ bound to S100B can be displaced with Zn$^{2+}$, suggesting that Zn$^{2+}$ and Cu$^{2+}$ share the same binding sites (Nishikawa et al., 1997). The variable cation binding properties of the S100 proteins are consistent with their diverse functions.

1.3.6 Target binding by S100 proteins

Three crystal structures of S100 proteins bound to target peptides have been reported. The structures of S100A10 (Rety et al., 1999) and S100A11 (Rety et al., 2000) in complex with annexin II and annexin I peptides respectively, and the binding of p53 regulatory peptide to S100B (Rustandi et al., 2000) have been solved. The binding of the annexin peptides was quite similar, which is remarkable, as there is no sequence similarity between the peptides. The p53 peptide bound to a different area of the same hydrophobic patch, in a nearly perpendicular orientation to that of the annexin peptides (indicated on the model of S100A6 in Figure 1.5). Therefore, the hydrophobic patch is thought to be a general target-binding site of S100 proteins and variations in sequence within the target binding regions are likely to account for the diversity of target interactions between family members. It has been suggested that full-length target proteins may in fact use a combination of the models of binding demonstrated for the S100 – peptide complexes (Otterbein et al., 2002).
Chapter 1: Introduction

There are a few exceptions to this common description: some S100s have lost their ability to bind either one or both of the Ca\(^{2+}\) ions while still adopting a Ca\(^{2+}\)-bound like conformation (S100A7; (Brodersen et al., 1999), S100A10; (Rety et al., 1999) and S100A11; (Rety et al., 2000)). S100A10 does not undergo a Ca\(^{2+}\)-dependent conformational change as it is in a permanent Ca\(^{2+}\)-bound conformation although it does not bind Ca\(^{2+}\). Instead, S100A10 interacts with another protein, calpactin heavy chain to form calpactin 1.

Another exception is calbindin D\(_{9k}\). It has only about 25% identity to most of the other S100s and has a shorter linker region and shorter N-terminal and C-terminal sequences. Consequently, calbindin D\(_{9k}\) does not dimerise in solution. The structure of calbindin D\(_{9k}\) in the Ca\(^{2+}\)-bound and free forms has been solved. There are only very small changes in the structure upon binding calcium, and in contrast to the other S100 family members, calbindin D\(_{9k}\) is thought to function as a Ca\(^{2+}\) buffer (Skelton et al., 1994).

1.3.7 Gene structure and genome localisation

The intron/exon organisation of S100 genes is highly conserved and suggests that these genes might have evolved by gene duplication from a single ancestral gene (Zimmer et al., 1996). With only a few exceptions, S100 genes consist of three exons separated by two introns, shown schematically in Figure 1.4. The first exon is not translated, the second exon encodes the N-terminal EF-hand and the third exon encodes the C-terminal EF-hand and 3' untranslated region.
Chapter 1: Introduction

A comparison of human S100 family cDNA sequences reveals that the average percent identity is 47%, with values ranging from 37% to 61% (Zimmer et al., 1996). The sequences of the promoter region of the genes and the 5' and 3' untranslated regions of their respective mRNAs display very little similarity (Zimmer et al., 1996). This heterogeneity may reflect different regulatory mechanisms for the expression of the individual S100 genes.

The majority of human S100 genes are located in a gene cluster on human chromosome 1q21 (Schafer et al., 1995). The only exceptions are S100B (21q22), S100P (4p16) and calbindin D9k (Xp22). Murine S100 genes have a similar clustered organisation, localised to a syntenic region on chromosome 3 (Ridinger et al., 1998). Within the human and murine S100 clusters, subgroups of S100 genes exist; the subgroups S100A3/A4/A5/A6 and MRP-8 (S100A8)/MRP-14 (S100A9) are found in both species. However, S100A1 and S100A13 are found associated in human but separated in the mouse S100 cluster, which is consistent with a rearrangement of this region during evolution (Ridinger et al., 1998).

Interestingly, human S100A10 and S100A11 are located 1.6 Mb apart from the main S100 cluster, just following the epidermal gene cluster that contains epidermal differentiation genes such as involucrin, as well as genes coding for profilaggrin and trichohyalin that contain S100-like domains. The close association of these genes suggests they may be coordinately regulated during epidermal differentiation (Schafer et al., 1995).
1.3.8 Expression of S100 proteins

Members of the S100 protein family are expressed in a tissue-specific and cell type-specific pattern (reviewed in (Zimmer et al., 1995)). Some S100 proteins are expressed in a variety of tissues, for example S100A1 is expressed in neurons, cardiomyocytes, brain, kidney, liver and testes (Du et al., 2002). Other S100 proteins have a very restricted tissue distribution, for example S100P is found only in the placenta (Becker et al., 1992). Individual cell types can contain multiple S100 proteins that are expressed at different levels. For example murine glial cells express high levels of S100B but much lower amounts of S100A1 and S100A6 (Nishiyama et al., 2002). The expression of some S100 proteins is inducible. For example S100A4 and S100A11 are upregulated in cardiomyocytes following chronic β-adrenergic stimulation (Inamoto et al., 2000). S100 expression is often regulated during differentiation (Zimmer et al., 1995). For example, S100A1 expression increases during skeletal muscle development. The levels of some S100 proteins are regulated during cell cycle progression (reviewed by (Donato, 1999)). For example S100A6 is maximally expressed during the progression from G₀ to G₁ and S100B is synthesized during the G₁ phase.

1.3.9 S100 expression in disease

Altered S100 gene expression is observed during a range of diseases. S100 proteins are often differentially expressed in neoplastic tissues and the human chromosomal region containing the S100 gene cluster, 1q21, is frequently rearranged in several tumours (Ilg et al., 1996). For example, S100A6 has a very restricted distribution in normal tissues but is highly expressed in a wide range of tumours, including breast,
colon and thyroid tumours. S100A2 is expressed by a subset of cells in normal skin, lung, kidney and prostate, but is down regulated in tumours in all these tissues (Ilg et al., 1996).

The localisation of the human S100B gene to the 21q22 region chromosome 21 suggested the possibility that overexpression of the protein in trisomy 21 could promote the development of Down syndrome (Griffin et al., 1989). However, patients with only partial trisomy 21, excluding the region where S100B is located, exhibited all the features of Down syndrome (Pangalos et al., 1992) and transgenic mice that overexpress human S100B are only slightly impaired in spatial learning (Gerlai et al., 1995). These findings suggest that overexpression of S100B may only play a minor role in determining the severity of the disease. Psoriasin (S100A7) was first isolated from psoriatic skin as it is highly upregulated in psoriatic keratinocytes (Madsen et al., 1991).

1.3.10 General functions of S100 proteins

The restricted distribution of S100 proteins suggests that these proteins must play specific roles in the tissues in which they are expressed. There is substantial evidence that S100 proteins function as Ca\(^{2+}\) sensors and a wide variety of target proteins have been shown to interact with S100 protein family members (for a recent review see (Donato, 2001)). In a number of these situations, Ca\(^{2+}\)-induced conformational changes in S100 proteins regulate their interaction with their target proteins. However in general, the functions of S100 proteins are poorly defined and as yet many in vitro interactions and functions of S100 proteins have yet to be confirmed in vivo.
Chapter 1: Introduction

**Intracellular functions of S100 proteins**

S100 proteins have been proposed to play diverse intracellular functions. Several S100 proteins have been reported to inhibit protein phosphorylation by interacting with kinase substrates and blocking access of the kinase. For example, S100B inhibited the phosphorylation of the tumour suppressor protein p53 by protein kinase C in a Ca^{2+}-dependent fashion (Baudier et al., 1992). A number of enzymes have been reported to be regulated by S100 proteins. For example, the myosin-associated giant kinase twitchin is activated > 1,000 fold by S100A1 binding to the autoregulatory sequence in its active site in a Ca^{2+} dependent manner (Heierhorst et al., 1996). Another example is S100β that stimulates membrane-bound guanylate cyclase in photoreceptor-bipolar neurones to produce cyclic GMP (Duda et al., 2002). S100A1 and S100B proteins have been implicated in Ca^{2+} homeostasis. For example S100A1 can directly bind to the ryanodine receptor, causing an increased open probability of this Ca^{2+} channel (Treves et al., 1997). Various S100 proteins have been shown to interact with constituents of the cytoskeleton (reviewed in (Donato, 2001)). For example S100A11 binds to F-actin, S100B associates with microtubules, S100A4 interacts with myosin heavy chain and MRP-8/14 (S100A8/9) interacts with intermediate filaments (see section 1.4.7). These observations suggest that S100 proteins may be important in regulating the dynamics of the cytoskeleton.

**Extracellular functions of S100 proteins**

A number of S100 proteins have been reported to have extracellular functions, although these proteins do not possess a signal sequence and the mechanism of their secretion is unknown. This includes a subset of S100 proteins that function as
chemotactic factors for a variety of cell types (see sections 1.3.11 and 1.5). A number of extracellular functions have been reported for S100B (reviewed by (Donato, 2001)). S100B is secreted by glial cells and has been shown to induce elevations in intracellular $\text{Ca}^{2+}$ in neurones to enhance their survival and promote neurite extension (Barger and Eldik, 1992). The effect of S100B is concentration dependent, as at higher concentrations S100B has been reported to stimulate apoptosis (Fulle et al., 1997).

### 1.3.11 Receptors

Cell surface receptors that may mediate the responses to S100 proteins have recently started to be identified. The receptor for advanced glycation end products (RAGE) is a scavenger-type receptor that belongs to the immunoglobulin superfamily. RAGE binds a variety of ligands, including advanced glycation end products (AGE) and amyloid fibrils that are associated with diabetic microvascular disease and Alzheimers disease respectively. Several members of the S100 protein family have been shown to bind RAGE, including S100A12 (EN-RAGE, MRP-6) (Hofmann et al., 1999), S100B and S100A1 (Huttunen et al., 2000). This has lead to the proposal that S100 proteins are the physiological ligands for RAGE (reviewed in (Schmidt et al., 2000)).

S100A12 has been shown to bind to RAGE on a variety of cell types in vitro including endothelial cells, macrophages and peripheral blood mononuclear cells. In these cells, RAGE is reported to signal via Nuclear Factor-$\kappa$B (NF-$\kappa$B) to stimulate a variety of pro-inflammatory responses including upregulation of adhesion molecules, chemotaxis, proliferation and cytokine generation (Hofmann et al., 1999). The
importance of RAGE in vivo was demonstrated using murine delayed-type hypersensitivity and colitis models. Soluble RAGE or antibodies to RAGE were able to suppress the inflammatory response, and antibodies to S100A12 were able to significantly attenuate the response (Hofmann et al., 1999). In summary, MRP-6-RAGE interactions play an important role in the pathogenesis of the inflammatory response.

S100B is a potent glial-derived neurotrophic factor. However only recently has its mechanism begun to be understood. S100B and S100A1 have been reported to activate RAGE that signals via NF-κB to induce neurite outgrowth and cell survival. The specificity of this response was demonstrated by cells that express a RAGE cytoplasmic domain deletion mutant are unresponsive to the S100 proteins (Huttunen et al., 2000).

As RAGE binds several S100 protein family members, it has been proposed to serve as general receptor for S100 proteins. However, it may not be responsible for the effects of every S100 protein. The chemoattractant activities of S100L and murine MRP-8 are sensitive to pertussis toxin, suggesting that these protein bind to G protein-coupled receptors (Cornish et al., 1996; Komada et al., 1996). In addition, MRP-8/14 has been shown to bind to the fatty acid transporter CD36 and heparin-sulphate glycosaminoglycans (see section 1.4.11).

1.3.12 Insights into S100 function from mouse models

Insight into the physiological function of S100 proteins has been gained from S100 protein-deficient mouse models. Studies of S100A1<sup>-/-</sup> mice support a Ca<sup>2+</sup> sensor role
for S100 proteins. S100A1<sup>−/−</sup> mice have reduced cardiac responses after β-adrenergic stimulation, associated with a reduced Ca<sup>2+</sup> sensitivity (Du et al., 2002). However a Ca<sup>2+</sup> buffer role for S100 proteins was suggested by studies of astrocyte cultures from S100B<sup>−/−</sup> mice. S100B<sup>−/−</sup> astrocytes from 6 day old mice display enhanced Ca<sup>2+</sup> transients upon stimulation, indicative of a buffering function of S100B (Xiong et al., 2000). However enhanced Ca<sup>2+</sup> transients were not observed in mature S100B<sup>−/−</sup> astrocytes in hippocampal slices in vivo (Nishiyama et al., 2002). An extracellular function of S100B is supported by the finding that S100B<sup>−/−</sup> mice display enhanced synaptic plasticity. This suggests that glial cell derived S100B can influence neuronal activity (Nishiyama et al., 2002). MRP-8<sup>−/−</sup> mice die in utero (Passey et al., 1999a), revealing an unexpected role for S100 proteins during embryonic development (see section 1.4.10).

1.4 The MRP proteins

1.4.1 MRP introduction

MIF-related protein-8 (MRP-8 (S100A8)) and MRP-14 (S100A9) were initially isolated as part of a complex using a monoclonal antibody directed against macrophage migration inhibitory factor (MIF) (Burmeister et al., 1986). The 8 and 14 refer to the electrophoretic mobility these proteins, although their actual molecular weights are 10.8 and 14 kDa respectively. MRP-8 and MRP-14 were first purified from myeloid cells in 1987 by Odink and coworkers (Odink et al., 1987). Other groups that subsequently isolated the MRP-8/14 heterodimer proposed a varied nomenclature for these proteins. MRP-8/14 was identified as the cystic fibrosis
antigen (CFAg) found at elevated levels in the serum of cystic fibrosis patients (Wilkinson et al., 1988). The two components of CFAg were named calgranulin A and B, corresponding to MRP-8 and MRP-14 respectively. The human leukocyte L1 antigen was also found to comprise MRP-8/14 (Dale et al., 1983). MRP-8/14 was subsequently named “calprotectin” to describe the antimicrobial properties of this calcium-binding protein complex (Steinbakk et al., 1990). MRP-8 and MRP-14 were designated S100A8 and S100A9 as part of a new nomenclature for S100 proteins (Schafer et al., 1995). Recently, another closely related S100 protein, S100A12 (MRP-6) has been identified. Human S100A12 shows highest homology to MRP-14 (46%) and MRP-8 (40%) (Ilg et al., 1996). However, a search of murine genomic sequences indicates that the mouse does not possess an S100A12 equivalent gene ((Manitz et al., 2003; Robinson, 2000)).

1.4.2 Protein structure

The crystal structures of human MRP-8 (Ishikawa et al., 2000), MRP-14 (Itou et al., 2002) and S100A12 (Moroz et al., 2001) have been solved and are all representative of the common fold of the S100 family. Monomers comprise two EF-hand motifs separated by a linker region and are arranged in an antiparallel orientation to form a dimer, stabilised by non-covalent interactions. Of all the structures available for Ca$^{2+}$ bound S100 proteins, the structure of MRP-8, MRP-14 and S100A12 are the most similar to each other (Figure 1.6) (Itou et al., 2002). The major structural differences are localised in the hinge region and C-terminus.
Figure 1.6 Overlay of the structures of MRP-14, MRP-8 and S100A12
Comparison of the structures of the human MRP-14 dimer (red and orange monomers) with human MRP-8 and human S100A12 (MRP-6) monomers (blue and yellow monomers respectively).
Figure reproduced from Itou et al., 2002.
In addition there are differences in the distribution of electrostatic potential and hydrophobicity between these proteins. For example the region surrounding the C-terminal EF-hand hinge region is strongly negatively charged in MRP-14, whereas the corresponding region in MRP-8 has a neutral potential and is largely hydrophobic. Therefore MRP-8/14 is likely to have differences in charge and hydrophobicity between the two halves of the heterodimer, allowing it to bridge different target molecules.

1.4.3 Structure of MRP-8

The structure of human MRP-8 in the Ca^{2+} bound form has been determined at 1.9 Å resolution by X-ray crystallography (Ishikawa et al., 2000). The electron density of the C-terminal EF-hand was higher than that of the N-terminal EF-hand, consistent with its higher affinity for Ca^{2+}, a characteristic feature of the S100 protein family.

In MRP-8, a glutamic acid residue that coordinates Ca^{2+} in the N-terminal EF-hand in most S100 proteins is replaced by an aspartic acid residue (Asp33), which has a much shorter side chain. The crystal structure shows that because of this change, the side chain O atom is not directly ligated to the Ca^{2+}, instead a water molecule forms a bridge between Asp33 and the Ca^{2+}.

Four isoforms of human MRP-8 have been detected in human neutrophil lysates (Guignard et al., 1996). One of these isoforms is phosphorylated in PMA-activated neutrophils. Phosphorylated MRP-8 constitutes only a small fraction of total MRP-8 and does not translocate to the plasma membrane upon cellular activation (see section 1.4.7) (Guignard et al., 1996).
Chapter 1: Introduction

1.4.4 Structure of MRP-14

A distinguishing feature of MRP-14 is its extended C-terminus, making it considerably larger than other S100 family members. The crystal structure of Ca$^{2+}$-bound human MRP-14 has been recently solved at 2.1 Å resolution (Itou et al., 2002) and shows that this region of the protein has a poor electron density, suggesting that has an extensively flexible character. This is consistent with the amino acid sequence that is comprised of largely hydrophilic residues and is not predicted to form a rigid secondary structure. This C-terminal tail region, specifically residues 90-112, is homologous to a “contact site” region within the serum protein, high molecular weight kininogen (HMWK) (see section 1.4.12) (Hessian et al., 1995). The C-terminal tail region of human MRP-14 also contains a stretch of amino acids (residues 89 – 108) that shows complete identity with the first twenty amino acids of neutrophil immobilising factor (NIF)-1 (Freemont et al., 1989). NIF-1 is reported to inhibit neutrophil random migration and chemotaxis by an unknown mechanism (Watt et al., 1983). However, the crystal structure of MRP-14 reveals that half of the NIF sequence, residues 85-94, participates in inter-monomer interactions, and is not available to interact with target molecules (Itou et al., 2002).

In the crystal structure of Ca$^{2+}$ bound human MRP-14, the detergent compound Chaps bound to a hydrophobic patch at the surface of the hinge region connecting the two EF-hand motifs. In the crystal structures of S100A10 (Retey et al., 1999) and S100A11 (Retey et al., 2000) in complex with their target annexin peptides, the target molecules bound to a similar hydrophobic patch. Therefore MRP-14 may interact with target proteins using a region that is thought to be a general target-binding site of S100 proteins.
Chapter 1: Introduction

Human MRP-14 exists as two isoforms due to the presence of an alternative start site, which results in the synthesis of a protein that is five amino acids shorter (Edgeworth et al., 1991). However, no difference in the expression, localisation or function of these isoforms has been reported. Both isoforms can be phosphorylated on the penultimate residue, threonine 113 (Edgeworth et al., 1989). In neutrophils and monocytes under resting conditions, only a small proportion of MRP-14 exists in the phosphorylated state. However, there is a large increase in the proportion of phosphorylated MRP-14 following stimulation with Ca\(^{2+}\) ionophores, such as ionomycin, Ca\(^{2+}\) dependent activators, such as fMLP and extracellular Ca\(^{2+}\) independent activators such as PMA (Edgeworth et al., 1989; Guignard et al., 1996). Unlike human MRP-14, murine MRP-14 has not been shown to be phosphorylated, and the corresponding threonine residue in the C-terminus is absent (Lagasse and Weissman, 1992). Four post-translational modifications have been identified for murine MRP-14: removal of the N-terminal methionine, N-terminal acetylation, disulphide bond formation between cysteine 79 and cysteine 90, and 1-methylation of histidine 106 (Raftery et al., 1996).

1.4.5 Complexes of MRP-8/14

Human MRP-8 and MRP-14 primarily exist as a non-covalently associated heterodimer with a stoichiometry of 1:1 (Edgeworth et al., 1991). Information from physical studies with the human proteins indicates that MRP-8 and MRP-14 can form homodimers, although the heterodimer is formed more readily than either homodimer (Hunter and Chazin, 1998). In addition MRP-8 in particular is unstable in the absence of MRP-14 (Hunter and Chazin, 1998). Human MRP-8 and MRP-14
can also form higher order complexes (Teigelkamp et al., 1991). Complex formation was studied by chemically crosslinking MRP-8 and MRP-14 that had been purified from myeloid cell lysates. A trimer and a tetramer corresponding to non-covalently associated \((\text{MRP-8})_2(\text{MRP-14})\) and \((\text{MRP-8})_2(\text{MRP-14})_2\) were isolated. The formation of these complexes was shown to be \(\text{Ca}^{2+}\)-dependent as the association was diminished in the presence of EDTA.

Murine MRP-8 and MRP-14 preferentially form heterodimers and murine and human MRP-14 can form heterodimers with MRP-8 from either species (Nacken et al., 2000; Propper et al., 1999). Using the yeast two-hybrid system, murine but not human MRP-8 and MRP-14 were also shown to form homodimers, although interaction to form heterodimers was preferred (Propper et al., 1999).

Using the yeast two-hybrid system, S100A12 (MRP-6) has been shown to form homodimers (Vogl et al., 1999). There is no evidence for an interaction of S100A12 with MRP-8 and/or MRP-14 to form a complex in human myeloid cells (Robinson and Hogg, 2000; Vogl et al., 1999).

1.4.6 Cation binding

\(\text{Ca}^{2+}\) binding affinities have yet to be measured for MRP-8/14, but other S100 proteins bind in the \(\mu\text{M}\) to \(\text{mM}\) range (Heizmann and Cox, 1998). MRP-8/14 has been shown to bind \(\text{Zn}^{2+}\). Excess \(\text{Ca}^{2+}\) did not antagonise \(^{65}\text{Zn}^{2+}\) binding to murine or human MRP-14, indicating that the \(\text{Zn}^{2+}\) binding site is distinct and independent from the two \(\text{Ca}^{2+}\)-binding domains (Raftery et al., 1996). The binding of \(\text{Ca}^{2+}\) and \(\text{Zn}^{2+}\) to human MRP-8/14 has been shown to induce distinct conformational changes.
in the heterodimer (Kerkhoff et al., 1999b). The binding of Ca\(^{2+}\) resulted in the exposure of hydrophobic residues, consistent with the Ca\(^{2+}\) induced conformational change seen in other S100 proteins for which the Ca\(^{2+}\) free and Ca\(^{2+}\) bound structures have been solved (see section 1.3.3).

1.4.7 Subcellular localisation

In resting myeloid cells, MRP-8 and MRP-14 are located predominantly in the cytosol (Edgeworth et al., 1991). Elevations in intracellular Ca\(^{2+}\) caused by the Ca\(^{2+}\) ionophore A23187 or stimuli such as opsonized zymosan, cause translocation of MRP-8 and MRP-14 to the membrane and vimentin type III intermediate filaments of the cytoskeleton (Burwinkel et al., 1994; Lemarchand et al., 1992; Roth et al., 1993). A similar translocation occurs in epithelial cells that express MRP-8/14. Stimulation of TR146 human squamous cell carcinoma cells with the Ca\(^{2+}\) ionophore, ionomycin, caused the translocation of MRP-8/14 from the cytoplasm to the keratin intermediate filament cytoskeleton (Goebeler et al., 1995b). Translocation of MRP proteins was also observed following ionophore stimulation of a transfected human embryonic lung cell line L132 expressing MRP-8 and MRP-14 alone or in combination (Roth et al., 1993).

Both phosphorylated and unphosphorylated isoforms of human MRP-14 can translocate from the cytosol to the membrane in myeloid cells. However there is disagreement whether phosphorylation serves to regulate this translocation (Guignard et al., 1996; van den Bos et al., 1996). Similar to MRP-8 and MRP-14, Ca\(^{2+}\) induces the translocation of S100A12 (MRP-6) to the membrane and cytoskeleton in human granulocytes (Vogl et al., 1999).
1.4.8 Gene structure and organisation

The MRP-8 and MRP-14 genes are single copy genes (Lagasse and Clerc, 1988), localised within the S100 gene cluster on human chromosome 1q21 (Schafer et al., 1995) and murine chromosome 3 (Ridinger et al., 1998). *MRP-8* and *MRP-14* genes are found in close proximity to each other and constitute a subgroup within the S100 gene cluster. In humans, the genes are separated by the closely related gene, *S100A12 (MRP-6)* (Wicki et al., 1996). The murine and human MRP-8 and MRP-14 genes have been cloned and have a three exon structure similar to other S100 genes (Lagasse and Clerc, 1988; May, 1999; Nacken et al., 1996). The formation of the truncated isoform of human MRP-14 is due to alternative translation of a single mRNA species and is not due to post-translational proteolysis (van den Bos et al., 1996).

1.4.9 Comparison of human and murine MRPs

At the protein level, both murine MRP-8 and MRP-14 share 59% identity with their human counterparts. The areas of greatest homology are located in the calcium-binding sites and the distance (but not sequence) between the two calcium-binding sites is very conserved between species (9 amino acids for MRP-8, 14 amino acids for MRP-14) (Lagasse and Weissman, 1992). Murine and human MRP-8 and MRP-14 protein sequences are more divergent than other members of the S100 protein family, which generally share at least 75 - 85% amino acid identity between vertebrates.
Chapter 1: Introduction

Using the yeast two-hybrid system, murine but not human MRP-8 and MRP-14 were shown to form homodimers, although interaction to form heterodimers was preferred (Propper et al., 1999). There are also differences in the post-translational modifications of MRP-14 in the two species (see section 1.4.4). However, despite these differences, murine and human MRP-14 are thought to be functionally homologous, based on biochemical data such as expression pattern, subcellular localisation and heterodimer formation (Nacken et al., 2000).

1.4.10 Expression of MRP proteins

The expression of MRP-8, MRP-14 and MRP-6 is restricted to cells of the myeloid lineage and certain epithelial cells. In addition, MRP-8 is expressed by an undefined population of cells during embryonic development.

Myeloid expression

In human neutrophils, MRP-8/14 comprises 45% of neutrophil cytosolic protein, which is equivalent to a concentration of approximately 3 mM (Edgeworth et al., 1991). In human monocytes, MRP-8/14 comprises approximately 1% of cytosolic protein (Edgeworth et al., 1991). MRP-8/14 is not expressed by eosinophils, platelets or lymphocytes (Hogg et al., 1989). Murine MRP-14 is also abundantly expressed, comprising 10 - 20% of neutrophil cytosolic protein (Nacken et al., 2000). Human MRP-6 is expressed in neutrophils and comprises 5% of the cytosolic protein (Guignard et al., 1995). Human monocytes express S100A12 (MRP-6), but at a lower level than neutrophils (Robinson and Hogg, 2000).
In human bone marrow, MRP-8/14 expression is first detected at the metamyelocyte stage of neutrophil development (Hessian et al., 1993). In murine bone marrow, MRP-8/14 expression overlaps with Mac-1 expression, suggesting that these cells are quite mature (Lagasse and Weissman, 1992).

Human resident tissue macrophages in general do not express MRP-8/14 (Hogg et al., 1989; Zwadlo et al., 1988), although macrophages in acutely inflamed tissues may express only MRP-14 (Delabie et al., 1990; Zwadlo et al., 1988). Similarly, the expression of MRP-8/14 by murine monocytes is lost as these cells terminally differentiate into tissue macrophages (Goebeler et al., 1995a; Lagasse and Weissman, 1992). Therefore the expression of MRP-8/14 is down-regulated as monocytes mature into macrophages. However, at sites of chronic inflammation, in patients with diseases such as rheumatoid arthritis or tuberculosis, macrophages do express MRP-8/14 (Odink et al., 1987; Zwadlo et al., 1988).

**Epithelial expression**

A subset of normal human squamous epithelia has been shown to express MRP-8/14. Squamous epithelia of mucous membranes such as the tongue, oesophagus and vagina/cervix, express MRP-8/14, with the exception of the cells in the basal layer that are normally negative (Brandtzaeg et al., 1987). MRP-8/14 is also expressed by hair follicles, in the medulla of the hair shaft. This expression overlaps with involucrin, suggesting that only terminally differentiated cells express MRP-8/14 (Schmidt et al., 2001). Normal human interfollicular epidermis and secretory epithelia such as salivary and pyloric glands, do not express MRP-8/14, but their expression is induced in diseases associated with increased proliferation of epithelial
cells (see section 1.4.10). In addition, cultured normal human keratinocytes and various epithelial cell lines express MRP-8/14, which is associated with terminal differentiation of these cells (Saintigny et al., 1992; Thorey et al., 2001). This may be due to the high rates of proliferation of these cells.

The epithelial expression of S100A12 (MRP-6) partially overlaps with MRP-14 staining, but appears to be more restricted. S100A12 is expressed with MRP-14 by the squamous supra-basal epithelial cells of the esophagus, but is absent from hair follicles in the epidermis (Robinson and Hogg, 2000).

**Expression during embryonic development**

MRP-8 mRNA has been reported to be expressed independently of MRP-14 mRNA, between day 6.5 and 8.5 of murine embryonic development (6.5 - 8.5 d.p.c.) by fetal cells infiltrating the maternal decidual tissue in the vicinity of the ectoplacental cone (Passey et al., 1999a). At 10.5 - 11.5 d.p.c., MRP-8 mRNA is detected in the placenta, expressed by maternal cells associated with the vasculature (Passey et al., 1999a). At later stages of murine development, MRP-8 and MRP-14 are coexpressed during hematopoiesis, in the yolk sac and fetal liver at increasing levels from 11 d.p.c., corresponding with the development of the myeloid lineage (Lagasse and Weissman, 1992).

**Extracellular expression**

MRP-8 and MRP-14 lack conventional signal sequences for secretion via the ER/golgi route and the mechanism by which they are released is poorly understood. One report suggests that the MRP proteins are secreted by monocytes through a
tubulin-dependent mechanism (Rammes et al., 1997). Human MRP-8/14 is detected extracellularly on the vascular endothelium of small venules at inflammatory sites adjacent to migrating myeloid cells (Figure 1.7) (Hogg et al., 1989; Robinson et al., 2002). As MRP-8/14 mRNA is not expressed by human endothelial cells (Robinson et al., 2002), the myeloid cells are the source of the deposited protein. A murine endothelial cell line has been reported to express MRP-8 mRNA (Yen et al., 1997), suggesting the possibility that murine endothelial cells may be a source of MRP proteins.

Expression associated with disease

MRP-8/14 is detected at elevated levels in the plasma of cystic fibrosis patients, and clinically normal heterozygotes as well as in patients with other chronic inflammatory diseases such as rheumatoid arthritis and sarcoidosis (Bullock et al., 1982). Inflammatory myeloid cells are likely to be the source of MRP-8/14 in these conditions.

MRP-8 and MRP-14 are highly expressed in a number of pathological conditions associated with an accelerated turnover and proliferation of epithelial cells. These include inflammatory dermatoses including psoriasis, eczematous dermatitis, lupus erythematosus and squamous cell carcinomas (Brandtzaeg et al., 1987; Gabrielsen et al., 1986; Kunz et al., 1992; Wilkinson et al., 1988). MRP-8/14 was most often detected in the upper and middle layers of the epidermis (the spinous and granular layers), but in some lesions the basal layer also stained positive for these proteins.
Figure 1.7 MRP-8/14 is localised on the vascular endothelium adjacent to migrating myeloid cells
Immunohistochemical staining of a section of rheumatoid synovium using mAb 5.5 to detect MRP-8/14.
This figure was adapted from (Hessian et al., 1993).
Chapter 1: Introduction

MRP-8 and MRP-14 have also been shown to be expressed by murine keratinocytes and myeloid cells in vivo during skin inflammation, wounding and all stages of carcinogenesis (Gebhardt et al., 2002; Thorey et al., 2001). Evidence that MRP-8 and MRP-14 expression was associated with keratinocytes and not secondary to the skin inflammation came from studies showing upregulation of MRP-8 and MRP-14 in the epidermis of activin-overexpressing transgenic mice, which develop a hyperproliferative and abnormally differentiated epidermis in the absence of inflammation (Thorey et al., 2001). In addition, the expression of MRP-8/14 by murine keratinocytes was reproduced in vitro using the PMK-R3 cell line (Gebhardt et al., 2002).

A new disorder of Zn\(^{2+}\) metabolism has been reported with recurrent infections, inflammation and hyperzincaemia that is associated with very elevated levels of plasma MRP-8/14 and normal levels of neutrophil turnover (Sampson et al., 2002). As almost all of the zinc was bound to MRP-8/14 it is possible that patients were affected by zinc deficiency.

1.4.11 Receptors

Several classes of MRP-8/14 receptors have been recently described, all of which are expressed by the vasculature. MRP-8/14 has been shown to bind to heparin-sulphate glycosaminoglycan (GAG) modifications present on proteoglycans on the surface of a wide range of cell types including the endothelial cell line, HMEC-1 (Robinson et al., 2002). The binding of MRP-8/14 to endothelial cells was mediated by the MRP-14 subunit and was dependent on the presence of Zn\(^{2+}\) and Ca\(^{2+}\) (Robinson et al.,
Chapter 1: Introduction

2002). MRP-8/14 has also been reported to bind to carboxylated glycans that are constitutively expressed on mammalian endothelial cells (Srikrishna et al., 2001). This interaction is less characterised. The interaction of MRP-8/14 with endothelial proteoglycans has been proposed to account for the immobilisation of the MRP-8/14 complex seen on the endothelium in vivo.

MRP-8/14 has been shown to bind to CD36 (Kerkhoff et al., 2001). CD36 is a cell surface glycoprotein present on monocytes, macrophages, platelets, adipose tissue and microvascular endothelial cells. CD36 binds a variety of ligands including thrombospondin, collagen, oxidised low-density lipoprotein, anionic phospholipids, apoptotic cells and fatty acids (Febbraio et al., 2001). As MRP-8/14 has been shown to bind arachidonic acid (see section 1.4.12), interaction with CD36 may facilitate the uptake of fatty acids by cells.

1.4.12 Functions of MRP-8/14

MRP-8 and MRP-14 have been long associated with the inflammatory process, and the primary role of these proteins is thought to be in the regulation of inflammation. In addition, a variety of other functions have been proposed for MRP-8/14 based on in vitro experiments and the study of MRP-8−/− mice.

Inflammation

There are many lines of evidence linking MRP-8/14 to inflammation. MRP-8/14 is highly expressed by cells of the innate immune system. MRP-8/14 constitutes 45% and 1% of the total cytosolic protein of neutrophils and monocytes respectively (see

69
Myeloid cell activation causes the relocation of MRP-8/14 from the cytoplasm to the plasma membrane and cytoskeleton (Burwinkel et al., 1994; Lemarchand et al., 1992; Roth et al., 1993), suggesting that these proteins may be important for regulating myeloid cell function. MRP-8 and MRP-14 are not expressed by resident tissue macrophages, but their expression is differentially induced during acute and chronic inflammation (see section 1.4.10) (Odink et al., 1987; Zwadlo et al., 1988).

Elevated levels of MRP-8/14 are found in the serum of patients with cystic fibrosis and chronic inflammatory diseases such as sarcoidosis and rheumatoid arthritis (Hessian et al., 1993). MRP-8/14 has been shown to be localized to the vasculature at inflammatory sites, suggesting that MRP-8/14 has an extracellular role in influencing leukocyte trafficking (Hogg et al., 1989; Robinson et al., 2002).

Murine MRP-8 has been characterised as a potent chemotactic factor for myeloid cells, with activity at $10^{-13} \text{M in vitro}$ (see section 1.5) (Lackmann et al., 1992; Lackmann et al., 1993). MRP-8 is has also been shown to induce the recruitment of neutrophils and monocytes in vivo (Devery et al., 1994; Lackmann et al., 1993).

**Human MRP-14 does not activate Mac-1**

R. Newton in this laboratory demonstrated that recombinant human MRP-14 (rhMRP-14) activated the leukocyte integrin Mac-1 on neutrophils (Newton and Hogg, 1998). RhMRP-14 also induced the expression of Mac-1 on T lymphoblasts and stimulated binding to the Mac-1 ligand, fibrinogen (Newton, 1997). However,
recently it has been shown that the rhMRP-14-induced adhesion is not mediated by Mac-1 and that the rhMRP-14 induced expression of Mac-1 and the mAb 24 epitope (a $\beta_2$ integrin activation reporter) were also artefact (Robinson, 2000).

**Fatty acid binding**

Arachidonic acid is a 20 C-atom polyunsaturated fatty acid. Cellular activation leads to the cleavage of arachidonic acid from the plasma membrane, which is rapidly metabolised. Cyclooxygenase or lipoxygenase metabolise arachidonic acid to generate prostaglandins and leukotrienes, which are potent eicosanoid lipid mediators (Funk, 2001). Recominit murine MRP-8/14 and purified human neutrophil and keratinocyte MRP-8/14, but not monomers or homodimers, bind arachidonic acid in a calcium-dependent manner (Kerkhoff et al., 1999a; Klempt et al., 1997; Siegenthaler et al., 1997). The interaction of MRP-8/14 with unsaturated fatty acids is specific, as saturated fatty acids such as palmitic acid and stearic acid as well as eicosanoids, were poor competitors (Kerkhoff et al., 1999a; Siegenthaler et al., 1997). There is disagreement as to whether MRP-8/14 can bind the monounsaturated fatty acid, oleic acid (Kerkhoff et al., 1999a; Siegenthaler et al., 1997). A study of point mutants of human MRP-14 in which conserved basic residues were replaced with alanine demonstrated that His103-His105 in the extended C-terminus represents the fatty acid binding region (Sopalla et al., 2002).

The MRP-8/14 complex represents the principle arachidonic acid binding protein in human neutrophils (Kerkhoff et al., 1999a). Stimulation of HL-60 cells led to the simultaneous secretion of MRP-8/14 and arachidonic acid. The majority of secreted arachidonic acid was bound to the protein complex (Kerkhoff et al., 1999a).
However whether arachidonic acid and MRP-8/14 are secreted in a concerted or independent manner remains unknown. The biological significance of MRP-8/14 binding to arachidonic acid is unclear. Intracellularly, MRP-8/14 is unlikely to serve as a reservoir for arachidonic acid as binding does not occur in the absence of Ca$^{2+}$. However, it may be involved in arachidonic acid transport or metabolism. Extracellular MRP-8/14-arachidonic acid complex may serve as a transport mechanism for arachidonic acid to move it to target cells to be metabolized. In support of this idea, MRP-8/14 has been reported to directly interact with the fatty acid transporter, CD36 (Kerkhoff et al., 2001). Interaction of an MRP-8/14-arachidonic acid complex with CD36 may accelerate dissociation of the complex and facilitate the uptake of fatty acids by the cells. In addition, extracellular Zn$^{2+}$ may facilitate dissociation of arachidonic acid from MRP-8/14, and may act as an additional release mechanism (Kerkhoff et al., 1999b).

**NADPH oxidase activation**

The NADPH oxidase consists of one membrane bound component, a $b$-type cytochrome and four cytosolic components, p40, p47, p67 and Rac (Babior et al., 2002). Cellular activation leads to the translocation of the cytosolic components to the membrane bound component to form a functional enzyme. Using a cell-free system, bovine MRP-8/14 has been shown to enhance the activation of NADPH oxidase, through enhancing the turnover of the cytochrome $b$, but not its affinity for NADPH or O$_2$ (Doussiere et al., 2001; Doussiere et al., 2002). Using coimmunoprecipitation and protein fractionation, MRP-8/14 was shown to associate with the p67 cytosolic oxidase component. MRP-8/14 has been proposed to serve as
a scaffold for the cytosolic components to promote their interaction with the cytochrome \( b \) component (Doussiere et al., 2002). Alternatively, arachidonic acid has been reported to activate the NADPH oxidase, and the role of MRP-8/14 may be to deliver bound arachidonic acid to the oxidase complex (Doussiere et al., 2002).

**Antimicrobial**

Extracellular MRP-8/14 was reported to have antimicrobial activity against bacteria and fungi (Steinbakk et al., 1990). It was found to inhibit the growth of *Candida* species and *Cryptococcus neoformans* at a concentration of \( 4 - 128 \, \mu g/ml \) and at concentration \( 2 - 4 \) times higher, it was fungicidal. The complex also inhibited the growth of *E. coli*, *Klebsiella* species, *S. aureus* and *S. epidermidis* at \( 64 - 256 \, \mu g/ml \).

It was subsequently demonstrated that this antimicrobial activity was a consequence of the ability of MRP-8/14 to bind Zn\(^{2+}\), causing Zn\(^{2+}\) deprivation (Santhanagopalan et al., 1995). *C. albicans* was cultured on media containing high concentrations of Zn\(^{2+}\) to increase the intracellular levels of this ion. These organisms were then shown to be resistant to growth inhibitory effects of MRP-8/14 (Santhanagopalan et al., 1995). In addition, the effect of MRP-8/14 could be overcome by adding Zn\(^{2+}\), and chelating Zn\(^{2+}\) was similarly effective in preventing *C. albicans* growth (Santhanagopalan et al., 1995). The Zn\(^{2+}\)-reversible antimicrobial activity of MRP-8/14 against *C. albicans* has also been shown using recombinant proteins (Sohnle et al., 2000).

Zn\(^{2+}\) is essential for the function of a variety of key enzymes (including the DNA and RNA polymerases). Therefore by chelating Zn\(^{2+}\), MRP-8/14 may prevent the growth of a range of microorganisms. The concentration of MRP-8/14 in abscess
fluid exceeds that required to inhibit microbial growth (Sohnle et al., 1991) and may be an important part of host defense.

**Inducer of cytostasis and apoptosis**

MRP-8/14 was identified as a cell growth inhibitory factor purified from rat peritoneal exudate cells (Yui et al., 1993; Yui et al., 1995). The complex was reported to have cytostatic and cytolytic effect on a broad range of cells including T and B lymphocytes and a variety of tumour cell lines at a concentration of around 50 - 100 μg/ml (2 - 4 μM) (Yui et al., 1993; Yui et al., 1995). The effect of MRP-8/14 could be blocked by the addition of Zn^{2+} (Yui et al., 1997). However it is unclear what the physiological relevance of a relatively non-specific myeloid-derived cytostatic/cytolytic factor is *in vivo*.

**Protection from oxidative damage**

Human and murine MRP-8 can be readily oxidised by reactive oxygen species, such as hypochlorite, to a disulphide-linked homodimer (Harrison et al., 1999). The mass of the homodimer was consistent with the presence of additional oxidised residues, possibly methionines. This oxidised homodimer was detected *in vivo* in lung lavage fluid of mice with endotoxin induced pulmonary injury (Harrison et al., 1999). Geczy and coworker propose that the preferential formation of oxidised MRP-8 has a protective role, to minimise the damage to other tissue proteins caused by oxidants at inflammatory sites.
Chapter 1: Introduction

Anticoagulant

The C-terminal tail region of MRP-14 is homologous to a "contact site" region within the serum protein, high molecular weight kininogen (HMWK) (Hessian et al., 1995). The "contact site" allows HMWK to bind anionic surfaces, to initiate the intrinsic coagulation cascade. MRP-14 tail sequences can mediate similar binding to anionic surfaces and can inhibit intrinsic coagulation in vitro (Hessian et al., 1995). This finding led to the suggestion that a function of MRP-14 may be to block fibrin formation at sites of leukocyte transendothelial migration.

Development

MRP-8 has been demonstrated to be expressed between 6.5 and 8.5 d.p.c. by fetal cells infiltrating the maternal decidual tissue in the vicinity of the ectoplacental cone (Passey et al., 1999a). The importance of MRP-8 expression during development is demonstrated by the embryonic lethality of MRP-8−/− mice that are resorbed by 9.5 d.p.c. (Passey et al., 1999a). The role of MRP-8 during development is not well understood. MRP-8 has been proposed to be involved in fetal-maternal interactions (Passey et al., 1999a).

Inhibitor of protein kinases

The MRP-8/14 complex has been shown to inhibit casein kinases I and II in myeloid cells with a $K_i$ value of $\leq 1 \mu M$. (Murao et al., 1988). By this mechanism, MRP-8/14 may indirectly regulate gene expression in myeloid cells.
Chapter 1: Introduction

1.5 S100 proteins as chemotactic agents

A growing number of S100 proteins have been reported to be chemotactic, suggesting that a subset of S100 proteins represent a new class of chemoattractants. Murine MRP-8 is also known as chemotactic protein of 10 kDa (CP-10), and was the first S100 protein to be reported to function as a chemotactic factor. Purified MRP-8 from the supernatants of ConA-activated murine spleen cells had a maximal chemotactic activity for murine neutrophils at $10^{-13}$ M (Lackmann et al., 1992), making it one of the most potent chemoattractants described to date. WEHI 265 monocytoid cells and human neutrophils also migrated towards murine MRP-8 with a maximal activity $10^{-11} - 10^{-12}$ M (Lackmann et al., 1993). Intradermal injection of CP-10 caused a sustained recruitment of neutrophils and monocytes into the skin over 24 hours, demonstrating that murine MRP-8 is also chemotactic in vivo (Devery et al., 1994; Lackmann et al., 1993). A synthetic peptide corresponding to the hinge region of CP-10 was chemotactic for the same cell types in vitro, and elicited a strong but more transient cell recruitment in vivo (Lackmann et al., 1993). The chemotactic activity was species specific as human MRP-8 and its corresponding hinge peptide were not chemotactic in vitro or in vivo (Lackmann et al., 1993). This may be accounted for by the low sequence similarity of murine and human MRP-8 hinge regions (21%) compared to their Ca$^{2+}$ binding domains (65%).

Chemotaxis stimulated by murine MRP-8 was inhibited by pertussis toxin, suggesting that it signals via a G-protein coupled pathway (Cornish et al., 1996). Murine MRP-8 caused a rapid an increase in actin polymerisation and altered cellular morphology (Cornish et al., 1996). Unlike classical chemoattractants, such as fMLP and IL-8, CP-10 failed to cause a Ca$^{2+}$ flux, superoxide burst, degranulation or the
Chapter 1: Introduction

coordinate up-regulation of Mac-1 and down-regulation of L-selectin (Cornish et al., 1996; Devery et al., 1994). Therefore, CP-10 can be classed as a non-classical chemoattactant, like TGF-β, that induces chemotaxis without cellular activation.

The chemotactic activity of CP-10 can be regulated by dimerisation. Reactive oxygen species generated at inflammatory sites in vivo oxidised the single Cys\(^{41}\) residue of MRP-8, to form a covalent homodimer (Harrison et al., 1999). The MRP-8 homodimer was inactive in chemotaxis assays in vitro and failed to recruit leukocytes in vivo. Therefore, dimerisation may block the exposure of the chemotactic hinge domain of MRP-8 and serve to limit the recruitment of leukocytes to an inflammatory site (Harrison et al., 1999).

Bovine S100A12 (MRP-6) has been reported to be chemotactic for human peripheral blood monocytes with an optimal activity at 1 μg/ml (= 1 nM). This was shown to be mediated by S100A12 binding to RAGE as the chemotactic activity could be blocked by pretreating the cells with F(ab')\(_2\) fragments to RAGE or by incubating the S100A12 with soluble RAGE prior to the chemotaxis assay (Hofmann et al., 1999). Human S10012 has also been reported to be chemotactic in vitro for monocytes and THP-1 cells with an optimal activity at 10\(^{-10}\) M, and neutrophils also responded very weakly. This did not mirror the ability of these cell types to respond to S100A12 in vivo. Neutrophils were the major leukocyte responding to a peritoneal injection of S100A12 at 8 hours, whereas monocytes were less responsive (Yang et al., 2001). This discrepancy may be due to an indirect mechanism of action of S100A12 in vivo; S100A12 has been shown to induce the production of cytokines by macrophages in vitro (Hofmann et al., 1999), which may cause the recruitment of neutrophils seem in vivo. In contrast to murine MRP-8, S100A12 stimulated a Ca\(^{2+}\)
Chapter 1: Introduction

flux in THP-1 cells (Yang et al., 2001). S100β has been shown to cause a Ca\(^{2+}\) flux in glial and neuronal cells (Barger and Eldik, 1992), therefore, there are differences in the signalling pathways induced by S100 proteins.

Other S100 proteins have been reported to function as chemoattractants but have been less extensively characterised. Human S100A7 is upregulated in psoriatic keratinocytes (Madsen et al., 1991) and has shown to be chemotactic for neutrophils and CD4\(^+\) T lymphocytes with an optimal activity at 10\(^{-11}\) M \textit{in vitro} (Jinquan et al., 1996). This chemotaxis was blocked with a neutralising antibody to S100A7. It remains to be tested whether S100A7 plays a role in the recruitment of these cell types \textit{in vivo}. Bovine S100A2 (S100L) has been shown to be chemotactic for guinea pig eosinophils with an optimal activity at 1 \textmu M (Komada et al., 1996). Similar to murine MRP-8, the chemotaxis induced by S100A2 was blocked by pertussis toxin, suggesting that S100A2 also signals via a G-protein coupled receptor.

1.6 Aims of this thesis

Just prior to the start of this thesis, Richard May in this laboratory demonstrated that recombinant MRP-14 (rMRP-14) was a potent chemoattractant for neutrophils and monocytes using the murine air pouch model (May, 1999). One aim of this thesis was to investigate the mechanism of how rMRP-14 was causing this myeloid cell infiltration.

A major objective was to perform basic characterisation of MRP-14\(^{+/−}\) mice that had been recently generated by Richard May in this laboratory, with the emphasis on examining myeloid cell function.
CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Stimulants, Inhibitors and other reagents

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Supplier</th>
<th>Stock solution</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHR</strong> (dihydrorhodamine)</td>
<td>Cambridge Bioscience</td>
<td>10 mM in DMSO</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>fMLP</strong> (N-formylmethionine-leucyl-phenylalanine)</td>
<td>Sigma</td>
<td>10 mM in Ethanol</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>Gliotoxin</strong></td>
<td>Sigma</td>
<td>2 mg/ml in Ethanol</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>Ionomycin</strong></td>
<td>Calbiochem</td>
<td>1 mM in DMSO</td>
<td>4 °C</td>
</tr>
<tr>
<td><strong>MIP-2</strong></td>
<td>Peprotech</td>
<td>0.1 mg/ml dH₂O</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>PdBu</strong> (Phorbol-12,13-dibutyrate)</td>
<td>Calbiochem</td>
<td>2 mM in DMSO</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>PMA</strong> (Phorbol-12-Myristate-13-acetate)</td>
<td>Calbiochem</td>
<td>2 mM in DMSO</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>SDF-1α</strong></td>
<td>Peprotech</td>
<td>0.1 mg/ml dH₂O</td>
<td>-20 °C</td>
</tr>
<tr>
<td><strong>Staurosporine</strong></td>
<td>Sigma</td>
<td>2 mM in DMSO</td>
<td>-20 °C</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and methods

### 2.1.2 Buffers/Serum

<table>
<thead>
<tr>
<th>Buffer/Serum</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium (DMEM; Sigma)</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum (FCS, Gibco-BRL)</td>
</tr>
<tr>
<td>V-LE FCS</td>
<td>Very low endotoxin FCS (Batch 122-323455, Autogen Bioclear)</td>
</tr>
<tr>
<td>H-HBSS</td>
<td>Hanks Balanced Salt Solution (HBSS; 10x; Gibco-BRL) containing 10 mM HEPES (1 M; Sigma)</td>
</tr>
<tr>
<td>FACSwash</td>
<td>PBS-A containing 0.2% bovine serum albumin (BSA; Sigma)</td>
</tr>
<tr>
<td>FAC斯fix</td>
<td>PBS-A containing 2% formaldehyde</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roosevelt Park Memorial Institute medium (RPMI; Gibco-BRL)</td>
</tr>
</tbody>
</table>

### 2.1.3 Antibodies and detection reagents for flow cytometry

<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>Conjugate</th>
<th>Epitope</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B10</td>
<td>FITC</td>
<td>MRP-14</td>
<td>Rat, IgG2a</td>
<td>CRUK</td>
</tr>
<tr>
<td>5C6</td>
<td>-</td>
<td>Mac-1, CD11b</td>
<td>Rat, IgG2b</td>
<td>CRUK</td>
</tr>
<tr>
<td>7/4</td>
<td>PE</td>
<td>Neutrophil/monocyte 40 kDa protein</td>
<td>Rat, IgG2a</td>
<td>Caltag Medsystems</td>
</tr>
<tr>
<td>B220 (RA3-6B2)</td>
<td>FITC</td>
<td>CD45</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>B220 (RA3-6B2)</td>
<td>CY-CHROMETM</td>
<td>CD45</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD4 (L3T4)</td>
<td>FITC</td>
<td>L3T4</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>
### Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th>CD8a (53-6.7)</th>
<th>PE</th>
<th>CD8 α and α’ chains</th>
<th>Rat, IgG2a</th>
<th>BD Biosciences</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80</td>
<td>Biotin</td>
<td>Macrophage 160 kDa glycoprotein</td>
<td>Rat, IgG2b</td>
<td>Serotec</td>
</tr>
<tr>
<td>Goat anti-rat IgG</td>
<td>FITC</td>
<td>-</td>
<td>Goat, polyclonal</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Gr-1 (RB6-8C5)</td>
<td>Biotin</td>
<td>Ly-6G/C</td>
<td>Rat, IgG2b</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Gr-1 (RB6-8C5)</td>
<td>FITC</td>
<td>Ly-6G/C</td>
<td>Rat, IgG2b</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IgG2a</td>
<td>-</td>
<td>-</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IgG2a</td>
<td>FITC</td>
<td>-</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>NK1.1 (PK136)</td>
<td>PE</td>
<td>NKR-P1B/C</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>Peridinin chlorophyll (PerCP)</td>
<td>-</td>
<td>-</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-CD16/32 (2.4G2)</td>
<td>-</td>
<td>FcyRII/III</td>
<td>Rat, IgG2b</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-CD62L (MEL-14)</td>
<td>Biotin</td>
<td>L-selectin</td>
<td>Rat, IgG2a</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Expression of rMRP-14

*E. coli* BL21 (DE3) plysS transformed with a construct comprising murine MRP-14 cDNA cloned into a pET-28a His-tag vector containing chloramphenicol and kanamycin resistance cassettes was a gift from R. May. A glycerol stock of the bacteria was streaked out onto LB agar containing 35 μg/ml chloramphenicol (Sigma) and 33 μg/ml kanamycin (Sigma) and grown overnight at 37 °C. A single colony was then used to seed a 20 ml overnight culture of LB media containing 35 μg/ml chloramphenicol and 33 μg/ml kanamycin. 8 ml of the overnight culture was then used to seed 500 ml of LB media with antibiotics. This culture was grown to an OD$_{600\text{nm}}$ of 0.6 - 1.0, when isopropyl-β-D-thiogalactopyranoside (IPTG; Amersham Biosciences) was added to a final concentration of 0.7 mM. The culture was grown for a further 4.5 hours, after which bacteria were pelleted at 3,800 x g and then frozen overnight at −20 °C. The thawed pellet was taken up in 25ml loading buffer (5 mM imidazole, 0.5 M NaCl, 20 mM TRIS-Cl pH 7.9) containing 2 μg/ml Phenylmethylsulfonyl fluoride (PMSF; Sigma) and 0.1% NP40. The suspension was then sonicated (Soniprep 150; Sanyo) 4 times for 30 sec on high power, and centrifuged at 100,000 x g for 1 hr. The supernatant was then filtered through a 0.45 μm filter and used as the starting material for protein purification.
2.2.2 Purification of rMRP-14

**Nickel column**

The rMRP-14 solution was loaded onto a 5 ml HiTrap Chelating column (Amersham Biosciences) that had been pre-equilibrated in loading buffer (5 mM imidazole, 0.5 M NaCl, 20 mM TRIS-Cl pH 7.9). The column was then washed extensively with loading buffer and non-specifically bound proteins were eluted with 5% elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM TRIS-Cl pH 7.9). The rMRP-14 was eluted by a gradient of 5 – 80% elution buffer over 60 ml. The fractions were analysed by SDS PAGE. Fractions containing high concentrations of rMRP-14 were pooled and diluted 1:5 in dH2O and Na2PO4 was added to a final concentration of 5 mM. The solution was then filtered through a 0.45 μm filter and used as the starting material for the hydroxyapatite column.

**Hydroxyapatite column**

The rMRP-14 solution was loaded onto a 5 ml hydroxyapatite column (Econo-Pac CHT-II cartridge; Bio-rad) that had been pre-equilibrated in loading buffer (5 mM Na2HPO4, 100 mM NaCl, 0.02% NaNz). The column was then washed extensively with loading buffer and non-specifically bound proteins were eluted with 5% elution buffer (500 mM Na2HPO4, 100 mM NaCl, 0.02% sodium azide). Bound proteins were eluted with 55% elution buffer. The fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Fractions containing high concentrations of rMRP-14 were pooled and dialysed overnight at 4 °C against H-
HBSS using 1 kDa dialysis tubing (Spectrum). Aliquots of protein were stored at -70°C and endotoxin was removed from aliquots prior to use in experiments.

2.2.3 Endotoxin removal and estimation

Detergent method

Endotoxin was removed by a phase separation method originally described by Aida and Pabst (Aida and Pabst, 1990). The protein solution was placed on ice and Triton X-114 (Sigma) was added to a final concentration 1%. The sample was then briefly vortexed and incubated on ice for 5 min. After vortexing again, the sample was placed at 37 °C for 5 min and centrifuged at full speed for 10 sec in a microfuge. The upper phase was then removed and used in assays.

KuttsuClean method

KuttsuClean (Maruha Corporation) is a cyclic agarose gel that is supplied as a slurry in sterile water. KuttsuClean was added at 1:10 to the protein solution and rotated overnight at 4 °C. The agarose was removed by centrifugation in a microfuge on a low setting for 7 minutes. The supernatant was decanted and used in assays.

Limulus Amoebocyte Lysate (LAL) Endotoxin Assay

Endotoxin was measured using the LAL endotoxin assay (Biowhittaker) according to the manufacturer's instructions. The assay was read using a Multiscan plate reader.
(Titertek) and the concentration of endotoxin in samples was calculated from the standard curve of endotoxin concentration versus absorbance readings.

2.2.4 Protein estimation

The concentration of rMRP-14 was determined using Bio-Rad protein assay dye reagent (Bio-Rad) according to the manufacturer’s instructions, using γ-globulin standards (0 - 500 μg/ml). The assay was read using a Multiscan plate reader (Titertek) and the concentration of test proteins calculated from the standard curve of protein concentration versus absorbance readings.

2.2.5 SDS PAGE

SDS PAGE analysis was performed essentially following the method of Laemmli (Laemmli, 1970). The polyacrylamide gel was composed of a stacking gel layered over a separating gel. The separating gel comprised of 375 mM Tris pH 8.0 containing 17.5% acrylamide and 0.47% bis-acrylamide (from an acrylamide/bis-acrylamide stock solution; Amersham Biosciences) and 0.1% SDS, 0.04% ammonium persulphate and 1/500 TEMED (Sigma). The stacking gel was composed of 125 mM Tris pH 6.8 with 3% acrylamide, 0.08% bis-acrylamide, 0.1% SDS, 0.04% ammonium persulphate and 1/500 TEMED. Proteins for analysis were boiled in sample buffer (125 mM Tris pH 6.8, 25% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.02% bromophenol blue) for 5 min just prior to loading onto the gel. Rainbow coloured protein molecular weight markers in the range 2 – 46 kDa (Amersham Biosciences) were run on each gel as standards. Electrophoresis was
performed in an Atto Dual Mini Slab Chamber (Genetic Research Instrumentation Ltd.) with electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 100 V through the stacking gel and 180 V through the separating gel. When required, proteins were visualised by staining with Coomassie Blue (0.5% Coomassie Blue (Bio-Rad) in 40% ethanol and 10% glacial acetic acid) and destained with a solution of 20% ethanol and 10% glacial acetic acid.

2.2.6 Two-dimensional electrophoresis

Bone marrow leukocytes were lysed at 1 x 10⁹/ml in 8 M urea, 4% 3-[(Cholamidopropyl)dimethylammonio]-1-propane-sulphanate (CHAPS) and 40 mM Tris base containing Complete, Ethylenediaminetetraacetic acid (EDTA) - free protease inhibitor cocktail (according to the manufacturer’s instructions; Roche), for 1 hr at room temperature (RT). The lysate was centrifuged at maximum speed in a microfuge and the supernatant was removed and diluted 1:4 in 8 M urea containing 2% CHAPS, 5% glycerol, bromophenol blue, 65 mM dithiothreitol (DTT) and 0.5% Immobilised pH Gradient (IPG) buffer. The lysate was separated by isoelectric focusing using an IPGphor™ 13 or 18 cm strip with a non-linear or linear pH range (pH 3 – 10), according to the manufacturer’s protocol (Amersham Biosciences). Protein spots were visualised using Colloidal Coomassie (17% ammonium sulphate, 0.1% Coomassie blue G-250, 0.5% acetic acid and 34% methanol). Protein spots on two groups of four 2D gels were compared using PDQuest 2D gel analysis software (Version 6.2; Bio-Rad). The Mann-Whitney test was used to test for proteins that were significantly altered between the groups.
Chapter 2: Materials and methods

2.2.7 Western blotting

Following SDS PAGE, proteins were transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) at 60 V for 50 min in a Transblot Cell (Bio-Rad) containing transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). The transfer was confirmed by staining the membrane with 0.1% Ponceau S solution (Sigma), then the membrane was blocked with PBS/Tween (PBS-A containing 0.1% Tween 20) containing 5% milk powder for 1 hr at RT or overnight at 4 °C. The membrane was then incubated with the primary antibody diluted in PBS/Tween containing 5% milk powder for 1 hr at RT. After washing three times in PBS/Tween, the membrane was similarly incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody then the membrane was washed again. Bound antibody was visualised by incubating the membrane with chemiluminescent substrate (ECL detection reagents; Amersham Biosciences) for 1 min, followed by exposing the membrane to film (Hyperfilm ECL; Amersham Biosciences). MRP-14 was detected with mAb 2B10 at 2 µg/ml compared to control IgG2a, followed by goat anti-rat HRP (1:5000, Southern Biotechnology). MRP-8 was detected using rabbit anti MRP-8 (1/1000) compared with control rabbit serum, followed by goat anti-rabbit HRP (1:2000, Dako-cytomation).

2.2.8 Chemokine Enzyme linked immunosorbent assays (ELISA)s

**KC, MIP-2, MIP-1β and MCP-1**

Levels of KC, MIP-2, MIP-1β and MCP-1 were analysed by ELISA according to the manufacturer’s instructions (R & D Systems Ltd.).
Chapter 2: Materials and methods

TNF-α

Maxisorp 96 well Nunc Immunoplates were coated overnight at RT with 100 μl/well capture antibody (R & D Systems) at 2 μg/ml in 0.1 M NaHCO₃ followed by washing three times with washing buffer (PBS containing 0.05% Tween 20 (Sigma)). Plates were blocked in blocking solution (PBS containing 0.1% BSA, 5% sucrose and 0.05% NaN₃) for 1 hr prior to the addition of 100 μl/well of samples or recombinant murine TNF-α standards (R & D systems) for 3 hr. The wells were washed, as above, before addition of 100 μl/well of detection antibody (R & D systems) diluted in blocking solution for 2 hr. Unbound antibody was removed by washing, and bound antibody detected with 100 μl/well streptavidin-HRP (R & D systems) diluted in blocking buffer for 20 min, followed by washing, then addition of 100 μl/well substrate solution (1:1 mixture of colour reagents A and B; R & D systems) for 1 hr in the dark. The reaction was stopped with 50 μl/well 1 M H₂SO₄ and the absorbance at 450 nm read on a Multiskan plate reader (Titertek). The concentration of TNF-α in test samples was calculated from the standard curve of TNF-α concentration versus absorbance readings.

2.2.9 Genotyping MRP-14⁺ mice

Isolation of DNA from mouse tails

Approximately 5 mm of tail was cut into an Eppendorf tube and 700 μl of tail buffer (50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS) was added. To each tube, 25 μl of a 10 mg/ml Proteinase K (Sigma) solution was added and tubes were
incubated at 55 °C overnight. 700 µl of phenol:chloroform (phenol:chloroform:isoamyl alcohol (25:24:1); Sigma) solution was added to the digested tail solution and tubes were vigourously shaken on a bench top shaker for 10 min. Tubes were centrifuged in a microfuge at full speed for 10 min. The top layer was transferred into a new tube and DNA was precipitated by the addition of 700 µl isopropanol. The DNA was pelleted by centrifugation at full speed in a microfuge for 15 minutes. The DNA pellet was then washed with 70% ethanol, allowed to air dry and dissolved in 50 µl water.

**Primers for polymerase chain reaction (PCR)**

<table>
<thead>
<tr>
<th></th>
<th>T&lt;sub&gt;m&lt;/sub&gt; °C</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF1</td>
<td>50.3</td>
<td>AACATCTGTGACTCTTTAGCC</td>
</tr>
<tr>
<td>GB1</td>
<td>50.3</td>
<td>CATCTGAGAAGGTGCTTTGTT</td>
</tr>
<tr>
<td>GNEO</td>
<td>56.2</td>
<td>ACCGCTTCCTCGTGCTTTACG</td>
</tr>
</tbody>
</table>

**PCR**

PCR reactions were set up in PCR plates (Cycleplate 24ET, Strip-ease-8 caps; Robbins Scientific) that contained 1 µl purified tail DNA, 125 ng of each primer, 1.5 U TAQ polymerase (Cancer Research UK) and PCR buffer (1x Thermophilic PCR buffer, 1.5 mM MgCl₂, 200 µM of dATP/dGTP/dCTP/dTTP; Promega) to a final volume of 25 µl. PCR programs were run on a Peltier Thermal Cycler PTC-225 (MJ Research) as described below:
2.2.10 Agarose gel electrophoresis

**Tris-acetate (TAE) buffer**

A 50 x stock solution was prepared by dissolving 242 g Tris base and 57.1 ml glacial acetic acid in dH₂O, then 100 ml 0.5 M EDTA, pH 8.0, was added and the final volume was made up to 1 l.

**DNA loading buffer**

A 10 x solution comprised 0.4% bromophenol blue (Sigma), 0.4% xylene cyanol FF (Bio Rad) and 50% glycerol in dH₂O.

**Electrophoresis**

TAE buffer containing 1.8 % agarose (Life technologies) was heated in a microwave oven to dissolve the agarose. Ethidium bromide was added (5 μl/100 ml; Sigma) to the agarose solution before it was cast in a mould. DNA loading buffer was added at...
Chapter 2: Materials and methods

1:10 to PCR reactions and 25 μl of each sample was added per agarose well. DNA was electrophoresed at 100 mV in 1 x TAE buffer.

2.2.11 Cell lines

**RAW 264.7 cells**

Murine RAW 264.7 monocyte/macrophage cells were maintained in RPMI with 10% FCS at 37 °C, 5% CO₂.

**WEHI 3B supernatant for myeloid cell culture**

Murine WEHI 3B myelomonocytic cells were maintained in RPMI with 10% FCS at 37 °C, 5% CO₂. WEHI 3B cells initially grew as a monolayer and daughter cells appeared in suspension. Cells in suspension were harvested when they reached a high density, resuspended in RPMI with 10% FCS and maintained for a further week. The conditioned media was collected, centrifuged to remove cell debris and stored at -20°C prior to use.

2.2.12 Cell preparations

**Murine bone marrow leukocytes**

Murine bone marrow leukocytes were harvested by flushing both femurs and tibiae with HBSS containing 0.2% BSA or the relevant assay buffer. Cell clumps were dispersed by gentle pipetting before the solution was filtered through a 100 μm cell
strainer (Falcon). Following centrifugation at 500 x g for 5 min, the cell pellet was resuspended in 1 ml erylse (0.144 M NH₄Cl/0.017 M Tris-HCl, pH 7.2) for three minutes to lyse the red blood cells, then the leukocytes were washed in DMEM containing 10 % FCS or the relevant assay buffer.

**Mature bone marrow granulocytes**

A Histopaque gradient was made by carefully layering 4 ml H-1077 (Sigma) on top of 4 ml H-1119 (Sigma). 1 ml of bone marrow leukocytes at 2-3 x 10⁷/ml (prepared as described above) in DMEM containing 10% FCS were then layered on top. Following centrifugation at 700 x g for 30 min, the granulocyte-rich band was collected from the interface of H-1119 and H-1077. Granulocytes were washed once in DMEM containing 10% FCS and resuspended at 2 - 3 x 10⁶/ml in DMEM containing 10% FCS and 10% WEHI 3B supernatant (see above). Cells were matured by overnight culture in Teflon pots (Tuf-tainers; Pierce) at 37 °C 5% CO₂.

**Murine blood mononuclear cells**

Mice were euthanised by carbon dioxide exposure and blood was taken by cardiac puncture and placed in tubes containing heparin (Sarstedt). Blood was diluted 1:4 with PBS-A and dextran (Pharmacia Biotech) was added to a final concentration of 1.2%. Erythrocytes were allowed to sediment for 45 min. A Histopaque gradient was made by carefully layering 4 ml H-1077 (Sigma) on top of 4 ml H-1119 (Sigma). Serum containing leukocytes from approximately 1 ml blood was then layered on top. Following centrifugation at 700 x g for 30 min, the mononuclear band at the top of the H-1077 was collected, then washed in the relevant assay buffer.
Chapter 2: Materials and methods

Preparation of detergent soluble leukocyte extracts

Leukocytes were suspended at $5 \times 10^7$/ml in ice cold lysis buffer (50 mM Tris pH 8.0 containing 1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 μg/ml PMSF and 1 μg/ml aprotonin) and incubated for 30 min on ice. The solution was then centrifuged at maximum speed in a microfuge to remove insoluble material and the supernatant decanted and used in assays.

2.2.13 Flow cytometry

Staining

Leukocytes were suspended at $5 \times 10^7$/ml in FACSwash and kept on ice, or at 30/37 °C where indicated.

Leukocytes were subjected to double immunostaining with mAb 7/4 and mAb Gr-1 in order to distinguish neutrophils (7/4 high, Gr-1 high) and monocytes (7/4 high, Gr-1 intermediate). The cells were incubated with PE-7/4 (1/20) and FITC-Gr-1 (1/200) for 20 min, then washed three times in FACSwash. Alternatively, the cells were stained with biotin-Gr-1 (1/50), washed in FACSwash, then stained with PerCP-Streptavidin (1/200) and PE-7/4 (1/20) and washed in FACSwash.

To identify individual classes of blood leukocytes, 50 μl blood was incubated with 50 μl saturating antibody solution (FITC-Gr-1 and PE-7/4, FITC-B220 and PE-NK1.1, FITC-CD8 and PE-CD4) for 30 min at RT. Cells were then washed in FACSwash and erythrocytes were lysed using FACSTM lysing solution (BD Biosciences) according to the manufacturer’s instructions.
Chapter 2: Materials and methods

To label cytosolic MRP-14, cells were placed on ice and fixed with 4% formaldehyde for 10 min then washed with FACSwash. Cells were then incubated with 0.1 M glycine in PBS for 10 min and washed with FACSwash. Following permeabilization with saponin buffer (0.5% saponin, 0.5% BSA and 10% FCS in PBS) containing 5 µg/ml anti-FcγRII/III to block non-specific antibody binding for 20 min, cells were labelled with mAb FITC-2B10, FITC-Phalloidin (Molecular Probes) or control mAb FITC-IgG2a, and washed with FACSwash.

Following staining with antibody, cells were resuspended in PBS-A containing 2% formaldehyde, or in assay buffer where indicated, and analysed using a FACScalibur (BD Biosciences).

**Absolute cell counting**

Absolute cell counting was performed by adding a known quantity of calibration beads (CaliBRITE™, BD Biosciences) to a known sample volume, then the number of cells were calculated as follows:

Total cells = (No. of cells counted x total no. of beads added)/(No. of beads counted)

**Cell sorting**

Cell populations that had been stained for flow cytometry were sorted using a Mo-Flo cell sorter (Dako-cytomation).
2.2.14 Immunohistochemistry

4 µm sections were cut from paraffin-embedded tissues and were microwaved for 10 min at 700 W. Sections were then stained with rat mAbs 2B10 (10 µg/ml) and IgG2a isotype control in Tris buffered saline, pH 7.6 for 40 min at RT, followed by biotinylated rabbit anti-rat Ig (1:100; Vector Laboratories) for 40 min at RT, then StreptABComplex/HRP used according to the manufacturer’s instructions (Dako-cytomation) and finally, exposure to 0.5 mg/ml of 3,3’-diaminobenzidine for 2 – 3 min. Slides were counter stained in Harris’ Haematoxylin.

2.2.15 In situ hybridisation and fluorescent Y chromosome staining of decidua

Preparation of decidua

Mating pairs were set up to generate 7.5 days post coitum (d.p.c) embryos. The timing of gestation was based on the assumption that coitus occurred at midnight on the evening before confirmation of a vaginal plug. Pregnant female mice were euthanised by carbon dioxide asphyxiation and uterine horns were dissected and placed in PBS. Individual decidua were separated from the uterus wall under a dissecting microscope using fine watchmakers forceps. Pairs of decidua were aligned in a petri dish containing a warm aqueous solution of 2% agarose, which was allowed to solidify. Squares of agarose containing the pairs of decidua were cut out and placed in neutral buffered formalin (NBF; 10% formalin, 45 mM Na₂HPO₄, 29 mM NaH₂PO₄·H₂O, pH 7) for 24 hr, then transferred to a 70% ethanol solution prior to embedding in paraffin.
Chapter 2: Materials and methods

**In situ hybridisation**

Specific localisation of the mRNA for MRP-8/14 was accomplished by *in situ* hybridisation using antisense riboprobes. The probes corresponded to the full length cDNA sequences of murine MRP-8 (343 bp coding for residues 1 – 89) and MRP-14 (488 bp coding for residues 1 – 113). Complementary RNA probe labelled with $^{35}$S-UTP (~800 Ci/mM; Amersham) was prepared as run-off transcript from *HindIII* linearised plasmid using T7 or SP6 RNA polymerase. An antisense β-actin probe was used as a control. All *in situ* hybridisation was performed on 4 μm serial sections of formalin-fixed, paraffin-embedded decidua (see above). The methods for pretreatment, hybridisation, washing and dipping of slides in photographic emulsion for autoradiography were as previously described (Senior et al., 1988). Autoradiography was carried out at 4 °C for 7 or 10 days before developing and counterstaining with Giemsa stain.

**Fluorescent Y chromosome staining**

To identify Y-chromosomes, sections were stained with a FITC-labelled Y-chromosome paint (Star-FISH, Cambio) as previously described (Poulsom et al., 2001).
Chapter 2: Materials and methods

2.2.16 Measurement of intracellular Ca$^{2+}$

Fluo-3

Cells were incubated at $10^7$/ml in Flux buffer (HBSS containing 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$, 10 µM Zn$^{2+}$, and 0.5% BSA) with 5 µM Fluo-3 (Molecular Probes) at 37 °C for 30 min. After two washes, the cells were resuspended at $2 \times 10^6$/ml in Flux buffer and maintained at 37 °C prior to use. 2 ml aliquots were added to quartz fluorimeter cuvets (Sigma) and stirred continuously. Fluorescence was monitored using a fluorimeter (Photon Technologies International; Excitation 485 nm, Emission 530 nm). Results represented as the emission at 530 nm.

Indo-1-acetoxyemthly ester (Indo-1-AM)

The measurement of intracellular Ca$^{2+}$ using Indo-1-AM was based on a published method (McColl and Naccache, 1997). Bone marrow leukocytes were incubated at $10^7$/ml in Flux buffer (HBSS containing 1 mM Ca$^{2+}$ and 0.5% BSA) with 2.5 µM Indo-1-AM (Molecular Probes) at 30 °C for 1 hr. After washing in Flux buffer, the cells were resuspended at $5 \times 10^7$/ml in Flux buffer and stained with mAbs PE-7/4 and FITC-Gr-1 at 30 °C for 15 min. Finally cells were washed and resuspended at $5 \times 10^6$/ml in Flux buffer and maintained at 30 °C prior to use. Fluorescence was monitored using a BD LSR™ flow cytometer (FL-4 530/30 nm BF filter, FL-5 424/44 nm filter; BD Biosciences). Samples were read at high flow rate (3000 events/sec) and stimulants were added at the indicated time points. Data was analyzed using FlowJo software (Tree star inc.). Results represented as the median Indo-1 ratio.
2.2.17 Chemotaxis assay

Migration assays were performed in Transwell plates with a 6.5 mm diameter and 3 μm or 5 μm pores (Corning) that had been preincubated with Chemotaxis buffer (H-HEPES containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, 10 μM Zn^{2+} and 0.1% BSA) at 37 °C for 1 hr. The bottom well was filled with 600 μl of various stimuli in Chemotaxis buffer and 100 μl of leukocytes at 3.5 - 5 x 10^6/ml in Chemotaxis buffer were placed in the top well. Migration was carried out at 37 °C for 2 - 4 hr, after which the Transwells were placed on ice and the top wells were discarded. The cells in the bottom well were collected and the wells washed with ice cold PBS-A containing 5 mM EDTA to collect any adherent cells. The cells were pelleted, stained with mAbs PE-7/4 and FITC-Gr-1 (see above) and resuspended in 200 μl FACSwash containing 10^4 calibration beads. The number of neutrophils and monocytes that had migrated was determined by flow cytometry.

2.2.18 Measurement of superoxide production

The measurement of superoxide production by flow cytometry was based on a published method and involves the conversion of di-hydrorhodamine (DHR) into a fluorescent derivative, rhodamine 123 (Smith and Weidemann, 1993). Prior to labeling with DHR, leukocytes at 5 x 10^7/ml in DMEM containing 10% FCS, were stained with Biotin-Gr-1 followed by PerCP-Streptavidin and PE-7/4 (see above). The cells were then resuspended at 2 x 10^6/ml in DMEM containing 10% FCS with 1 μM DHR at 37 °C for 5 min prior to exposure to stimuli. After 30 min stimulation at
Chapter 2: Materials and methods

37 °C, the samples were transferred to ice, and the production of superoxide by neutrophils and monocytes was measured by flow cytometry.

2.2.19 Apoptosis assay

Bone marrow leukocytes at $2.5 \times 10^6$/ml in DMEM containing 10% FCS were incubated at 37 °C together with stimuli to induce apoptosis (ionomycin, thapsigargin, staurosporine, gliotoxin or TNF-α) for 0 – 8 hr. Alternatively, spontaneous death in culture was examined by incubating bone marrow leukocytes at $2.5 \times 10^6$/ml in DMEM alone or DMEM supplemented with 10% FCS for 0 – 4 days in Teflon pots (Tuf-tainers; Pierce). To assess apoptosis in neutrophils and monocytes, cells were first stained with PE-7/4 and FITC-Gr-1 then resuspended in PBS containing 0.1 μM LDS 751 (Exciton) for exactly 20 min. 4',6-diamidino-2-phenylindole (DAPI; Sigma) was added to a final concentration of 0.1 μg/ml and samples were analyzed using a BD LSR™ flow cytometer (Becton Dickenson).

2.2.20 Air pouch model of inflammation

The protocol for this assay was based on that described by Tessier et al. (Tessier et al., 1997). Briefly, on day 0 and day 3, mice were anaesthetised with halothane and injected subcutaneously on the back with 2.5 ml sterile air to generate a dorsal air pouch. On day 6, the mice were anaesthetised and 1 ml of stimulus was injected. The stimulus was prepared in PBS-A containing 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$ and 10 μM Zn$^{2+}$. When endotoxin-extracted rMRP-14 was used as a stimulus, a solution of endotoxin-extracted H-HBSS equivalent to that present in the maximum dose of
rMRP-14 was used as a negative control. For antibody blocking experiments, intact antibodies were dialysed into PBS, concentrated and injected into the air pouch alone or in combination with rMRP-14. At various time points up to 6 hr, mice were euthanised by carbon dioxide exposure and cells that had migrated into the air pouch were collected by washing, once with 1 ml, and twice with 2 ml, of PBS-A containing 5 mM EDTA. The cells were counted in duplicate using a haemocytometer. Four to six mice were analysed at each time point.

2.2.21 Thioglycollate-induced peritonitis

Peritonitis was induced by intraperitoneal injection of 0.5 ml sterile thioglycollate (3% w/v in saline; Sigma), as described by Henderson et al. (Henderson et al., 2001). At various time points up to 72 hr, mice were euthanised by carbon dioxide exposure and peritoneal cavities were washed with 5 ml PBS-A containing 3 mM EDTA. Leukocytes were counted and the proportion of neutrophils, monocytes and other leukocytes was determined by flow cytometry. Six to eight mice of each genotype were analysed at each time point.

2.2.22 Streptococcus pneumoniae-induced pneumonia

Mice were lightly anesthetised with 1.5% (vol/vol) halothane and 50 μl of PBS containing 1 x 10^6 colony forming units of S. pneumoniae (D39, serotype 2) was then administered into the nostrils of each mouse as previously described (Kerr et al., 2002). At certain time points, mice were sacrificed by cervical dislocation and blood samples were taken via cardiac puncture. Lung tissue was removed and homogenized.
and viable bacterial counts were determined as previously described (Kerr et al., 2002). Four to six mice of each genotype were analysed at each time point.
CHAPTER 3

Effect of recombinant MRP-14 \textit{in vitro} and \textit{in vivo}

3.1 Introduction

There is accumulating evidence for a role for MRP-8/14 in inflammation. Firstly, the complex is highly expressed by cells of the innate immune system: neutrophils, monocytes and macrophages at sites of chronic inflammation (Hessian et al., 1993). Secondly, elevated levels of MRP-8/14 are found in the serum of patients with cystic fibrosis and chronic inflammatory diseases such as sarcoidosis and rheumatoid arthritis (Hessian et al., 1993). Thirdly, extracellular MRP-8/14 has been shown to be localized to the vasculature at inflammatory sites, adjacent to migrating inflammatory myeloid cells (Hogg et al., 1989; Robinson et al., 2002) suggesting that these proteins may influence leukocyte trafficking. Finally, murine MRP-8 (also known as CP-10) has been characterised as a potent chemotactic factor for myeloid cells with activity at $10^{-13} \text{M in vitro}$ (Lackmann et al., 1992; Lackmann et al., 1993).

A growing number of S100 proteins, including S100A12 (Hofmann et al., 1999), psoriasin (S100A7) (Jinquan et al., 1996) and S100L (S100A2) (Komada et al., 1996) have been reported to be chemotactic, suggesting that a subset of S100 proteins represent a new class of chemoattractants.

Using the air pouch model of inflammation, R. May, in this laboratory, demonstrated that recombinant MRP-14 (rMRP-14) was a potent chemoattractant for leukocytes \textit{in vivo} (May, 1999). The dose response curve was bell shaped, with a...
maximum response at 50 μg. rMRP-14 induced a strong leukocyte infiltrate within 3
hours, which peaked at 6 hours and decreased to almost background levels by 24
hours. The population of leukocytes at 6 hours comprised 70% neutrophils and 30%
monocytes. The aim of this project was to investigate the mechanism of how rMRP-
14 was causing myeloid cell infiltration into the air pouch.
3.2 Results

3.2.1 Expression and purification of rMRP-14

In order to investigate the biological activity of rMRP-14 seen in vivo, the effect of rMRP-14 was studied in vitro using a number of myeloid functional assays. For these assays, rMRP-14 was made in the same way as for the previous in vivo studies, using a bacterial expression system. rMRP-14 was expressed as a His-tagged protein using E. coli BL21 (DE3) plysS transformed with a pET-28a vector into which murine MRP-14 cDNA had been cloned (a gift from R. May). rMRP-14 was produced with an N-terminal Histidine (His) tag, with six consecutive His residues, to aid purification. This results in an increase in its molecular weight from 14 kDa to approximately 16 kDa. Expression of rMRP-14 was induced by addition of IPTG for 4.5 hours. MRP-14 was predominantly expressed as a cytosolic protein (May, 1999), therefore a cytosolic extract was prepared. The bacteria were lysed by freeze/thaw and sonication, then centrifuged to remove insoluble and aggregated material. SDS PAGE analysis of the resultant solution showed rMRP-14 to be a major constituent (Figure 3.1, Lane L).

rMRP-14 was purified by Fast Protein Liquid Chromatography (FPLC) using a Nickel column, to capture the His-tagged protein. Bound rMRP-14 was eluted with an ascending gradient of imidazole (Figure 3.1).
Figure 3.1 Nickel purification of rMRP-14 from bacterial lysate
A) The absorbance at 280 nm (black line) of the flow through of the nickel column and the percentage elution buffer (red line; see Material and Methods) during purification of rMRP-14.
B) SDS PAGE analysis of the purification of rMRP-14. rMRP-14 (indicated with an arrow head) was a major constituent of the bacterial cytosolic extract applied to the column (Lane L). rMRP-14 bound well to the Nickel column, indicated by the lack of rMRP-14 in the run through from the column during loading of the sample (Lanes R1 and R2). A large proportion of contaminating protein was removed from the column with a low stringency wash with 5% elution buffer (Fractions 8 –10). Bound rMRP-14 was eluted with an ascending gradient of imidazole. Fractions containing high concentrations of rMRP-14 (Fractions 55 - 61) were pooled.
M, Markers; L, Load; R1/2, Run through during column loading; white arrow head, rMRP-14.
This first purification step was very successful at removing the majority of the contaminating proteins from rMRP-14. Fractions containing high concentrations of rMRP-14 were pooled and further purified using a hydroxyapatite column. Hydroxyapatite resin binds, amongst other macromolecules, calcium binding proteins, and was used to remove any remaining bacterial impurities as well as misfolded non-calcium binding recombinant protein. Correctly folded rMRP-14 was eluted with high concentrations of phosphate, which migrated largely as a single band in SDS PAGE (Figure 3.2). Fractions containing high concentrations of rMRP-14 were pooled and dialysed into HEPES buffered HBSS, and frozen at -70 °C in aliquots.

Lipopolysaccharide (LPS) is a component of the Gram negative bacterial cell wall, and as a potent inflammatory mediator, can exert a range of biological effects in vivo, from fever to circulatory failure (“shock”). When preparations are contaminated with LPS, complexes of LPS, protein and phospholipid are produced, and the term endotoxin is used (Henderson and Wilson, 1996). To avoid contamination of purified rMRP-14, endotoxin was always removed prior to use of rMRP-14 in in vitro or in vivo assays, using two different methods. Initially, endotoxin was removed using Triton X-114, based on a published method (Aida and Pabst, 1990). The main disadvantage of this procedure is that a small amount of detergent can persist in the purified preparation, which could have unwanted biological effects (Aida and Pabst, 1990). However as a control for this, rMRP-14 was always used in assays alongside a detergent-extracted buffer control.
Figure 3.2 Hydroxyapatite purification of rMRP-14 from a semi-pure preparation

A) The absorbance at 280 nm (black line) of the flow through of the hydroxyapatite column and the percentage elution buffer (red line; see Material and Methods) during purification. rMRP-14 was already partially purified using a Nickel column.

B) SDS PAGE analysis of the hydroxyapatite column chromatography purification of rMRP-14. rMRP-14 (indicated with a white arrow head) was a major constituent of the solution applied to the column (Lane L). A substantial amount of rMPR-14 was removed from the column with a low stringency wash with 5% elution buffer (Fractions 6 - 14), this protein was likely to be mis-folded. Bound rMRP-14 was eluted with 55% elution buffer. Fractions containing high concentrations of rMRP-14 (Fractions 28 - 31) were pooled. M, Markers; L, Load; R, Run through during column loading; Fractions 6 - 37; arrow head, rMRP-14.
Subsequently, to avoid problems associated with detergent contamination of rMRP-14, a protocol for removing endotoxin using KuttsuClean was used. KuttsuClean has a high affinity for endotoxin and consists of a protein isolated from the amebocytes of the horseshoe crab (*Limulus polyphemus*) coated on the surface of agarose beads. Purified rMRP-14 was used in assays alongside a buffer control that had been similarly prepared using KuttsuClean. The biological activity of rMRP-14 in the air pouch was retained following endotoxin removal by either of the above protocols (May, 1999) and data not shown). Residual endotoxin contamination in purified rMRP-14 was measured using the Limulus Amoebocyte Lysate Assay. Following endotoxin removal using the Triton X-114 or KuttsuClean protocols, the level of endotoxin in 50 µg rMRP-14 was approximately 0.4 ng.

### 3.2.2 Purification and maturation of murine bone marrow granulocytes

As murine blood contains only 5 – 10% neutrophils, and the volume of blood recovered from a mouse is only ~1 ml, the total yield of neutrophils per mouse is very low, ~5 x 10^5. Therefore, for *in vitro* assays, an alternative source of neutrophils was investigated to minimise the numbers of animals used. A method for culturing bone marrow neutrophils was developed, based on a previously described method (Blanks et al., 1998). Granulocytes were separated from bone marrow cells using a Histopaque gradient, and were shown to be 75 – 85% pure by mAb 7/4 staining (data not shown). Granulocytes were then cultured overnight in DMEM supplemented with WEHI 3B conditioned media containing IL-3. This allowed the cells to mature, as shown by their ability to respond to fMLP. In agreement with Blanks *et al.* (Blanks et al., 1998), mature bone marrow granulocytes, but not freshly isolated
Chapter 3: Effect of rMRP-14 in vitro and in vivo

ones, could respond to fMLP as demonstrated by Mac-1 upregulation (Figure 3.3). Mature bone marrow granulocytes also shed L-selectin in response to fMLP (Figure 3.3). Mature bone marrow granulocytes prepared in this manner were used in in vitro assays to investigate the function of rMRP-14 seen in vivo.

3.2.3 rMRP-14 does not directly cause chemotaxis of myeloid cells

The ability of rMRP-14 to directly cause migration of leukocytes was tested in vitro using the Transwell chemotaxis assay. In this assay, a stimulus is placed in the bottom well of a 24 well tissue culture plate, which is separated from cells in the top well by a filter containing pores through which the cells can migrate. After a number of hours, the cells in the bottom well are counted and stained for flow cytometry. rMRP-14 was titrated across a wide range of concentrations to include the μM concentrations over which rMRP-14 is active in the air pouch down to the reported pM levels at which murine MRP-8 was reported to be chemotactic (Lackmann et al., 1992). rMRP-14 failed to stimulate migration of mature bone marrow granulocytes (Figure 3.4 A), or peripheral blood monocytes (Figure 3.4B) and lymphocytes (Figure 3.4 C). The ability of cells to migrate towards the chemokines MIP-2 or SDF-1 was included as a positive control. To test whether MRP-14 could act cooperatively with chemokines in inducing migration, the migration of granulocytes towards MIP-2 in the presence or absence of rMRP-14 was examined. No enhancement in the migration of granulocytes induced by MIP-2 was seen with addition of 1 μM MRP-14 (Figure 3.4 D).
Figure 3.3 “Mature” but not “immature” bone marrow granulocytes are responsive to fMLP

Bone marrow cells were flushed out of femurs and tibias of 8-10 week-old mice and granulocytes were separated using a Histopaque gradient. “Immature” granulocytes were recovered at the interface of the Histopaque 1077 and 1119 phases. Granulocytes were then cultured overnight in DMEM containing 10% FCS and 10% WEHI 3B conditioned supernatant to produce “mature” cells. Immature and mature granulocytes were incubated for 20 min with 1 μM fMLP and the expression Mac-1 and L-selectin was measured by flow cytometry.
Figure 3.4 rMRP-14 does not directly cause leukocyte chemotaxis
A) Chemotaxis of mature bone marrow granulocytes in Transwells containing 3 μm pores, in response to rMRP-14 or MIP-2 (10 ng/ml). Cells were allowed to migrate for 2 hours. Data representative of 4 experiments.
B) Chemotaxis of peripheral blood monocytes in Transwells containing 5 μm pores, in response to rMRP-14 or SDF-1 (10 ng/ml). Cells were allowed to migrate for 3 hours. Data representative of 2 experiments.
C) Chemotaxis of peripheral blood lymphocytes in Transwells containing 5 μm pores, in response to rMRP-14 or SDF-1 (10 ng/ml). Cells were allowed to migrate for 3 hours. Data representative of 2 experiments.
D) Chemotaxis of mature bone marrow granulocytes in Transwells containing 3 μm pores, in response to MIP-2 alone or in combination with 1 μM rMRP-14. Data representative of 2 experiments.
All data are expressed as mean ± SD.
3.2.4 rMRP-14 does not cause myeloid cell activation

Activation of leukocytes by chemotactic factors generally leads to a rapid and transient increase in the intracellular levels of free Ca$^{2+}$, known as a Ca$^{2+}$ flux. To test whether rMRP-14 caused a Ca$^{2+}$ flux in mature bone marrow granulocytes, levels of intracellular Ca$^{2+}$ were monitored using Fluo-3, a fluorescent Ca$^{2+}$ binding dye. As positive controls, fMLP a bacterial chemoattractant, and thapsigargin an inhibitor of the Ca$^{2+}$ ATPase on intracellular Ca$^{2+}$ stores, were used. Both fMLP and thapsigargin increased intracellular Ca$^{2+}$ levels and Fluo-3 fluorescence (Figure 3.5 A). However, rMRP-14 at concentrations up to 1 μM failed to cause a Ca$^{2+}$ flux in granulocytes (Figure 3.5 A). rMRP-14 also failed to cause a Ca$^{2+}$ flux in the monocytic THP-1 cell line (data not shown).

Superoxide production by the NADPH-oxidase is a measure of myeloid cell activation (Dahlgren and Karlsson, 1999). In a resting cell, the oxidase is inactive and consists of one membrane bound and four cytosolic components. Upon activation, the cytosolic components translocate to the membrane to form a functional enzyme, which leads to production of reactive oxygen species (Babior et al., 2002). To test whether rMRP-14 caused activation of the NADPH oxidase, cells were incubated with dihydrorhodamine (DHR). When oxidised, DHR increases its fluorescence, which can then be detected by flow cytometry. The positive controls, fMLP and phorbol ester (PDBu) both caused oxidase activation and a 4- and 20-fold increase in DHR fluorescence respectively (Figure 3.5 B). However, rMRP-14 failed to cause oxidase activation (Figure 3.5 B).
Figure 3.5 rMRP-14 does not cause a Ca\textsuperscript{2+} flux or superoxide burst in mature bone marrow granulocytes.

A) The ability of rMRP-14, fMLP (1 μM) or thapsigargin (5 μM) to cause a Ca\textsuperscript{2+} flux in mature bone marrow granulocytes. Cells were loaded with Fluo-3 and the fluorescence was monitored using a spectrofluorimeter. Data representative of 4 experiments.

B) The ability of rMRP-14, PDBu (200 nM) or fMLP (1 μM) to cause a superoxide burst in mature bone marrow granulocytes. Cells were loaded with DHR and 30 minutes after the addition of stimuli, fluorescence was measured by flow cytometry. Inset numbers are geomean fluorescences. Data representative of 3 experiments.
3.2.5 rMRP-14 induces leukocyte recruitment through an indirect mechanism

To test whether rMRP-14 indirectly caused leukocyte influx into the air pouch, the levels of cytokines in air pouch exudate were analysed. 50 μg of rMRP-14 was injected into the air pouch and cytokine levels in pouch fluid were measured at a series of time points between 0 - 6 hours. The levels of five cytokines were examined, including four chemokines, known to attract myeloid cells. The levels of the CXC chemokines, MIP-2 and KC, known to be chemotactic for neutrophils, and the CC chemokines, MIP-1α and MCP-1, chemotactic for monocytes, were examined. In addition, the levels of TNF-α were measured. TNF-α is essential for the recruitment of leukocytes to extravascular sites through its actions of regulating the expression of adhesion molecules and the production of chemotactic factors (Tessier et al., 1993; Tessier et al., 1997).

rMRP-14 caused a rapid increase in the level of MIP-2, KC, MIP-1α, MCP-1 and TNF-α within 30 minutes (Figure 3.6). The expression of cytokines was transient, following a peak at 1 hour, their expression fell to background levels by 6 hours. MIP-2, KC and TNF-α expression followed a similar kinetic profile, after peaking at 1 hour the levels of these cytokines rapidly decreased by 2 hours. This is in contrast to MIP-1α and MCP-1 whose expression remained elevated at 2 hours. As a positive control, the cytokines induced by TNF-α were examined. TNF-α induced KC, MIP-2 and MCP-1, with a similar kinetic profile to rMRP-14, however, TNF-α failed to induce MIP-1α and TNF-α expression.
Figure 3.6 The induction of cytokines in air pouch exudate by rMRP-14
rMRP-14 (50 μg; ▲), TNF-α (10 ng; ■) or buffer alone (O) were injected into
the air pouch and cytokines in air pouch exudate were measured by ELISA
at a series of time points.
These data are representative of 2 experiments which utilised 3 - 5 mice per
time point for each stimulus. Data reported are as mean ± SEM.
Chapter 3: Effect of rMRP-14 in vitro and in vivo

To investigate the source of the chemokines, immunohistochemistry was performed on air pouch tissue, one hour after rMRP-14 injection. This work was performed in collaboration with Philippa Munson at University College London. MIP-2 was widely expressed in air pouch tissue, by cells within and beneath the epithelial lining of the air pouch (Figure 3.7). A large proportion of these cells were identified as macrophages, based on their staining with mAb F4/80 (Figure 3.8 A). Neutrophils and monocytes were identified by staining air pouch tissue with mAb 7/4. Small number of these myeloid cells were seen, often in the vicinity of blood vessels (Figure 3.8 B). In summary, rMRP-14 induces the rapid expression of a variety of cytokines in the air pouch, and resident macrophages are a major source of the MIP-2 produced.

3.2.6 The effect of rMRP-14 in vivo can reproduced in vitro

To further investigate the induction of chemokines by rMRP-14 in vivo, an in vitro assay was developed using murine monocyte/macrophage cell lines. RAW 264.7, WEHI3 and P388 cells were incubated with medium alone, 50 µg/ml rMRP-14 or LPS as a positive control and the levels of TNF-α were measured in the culture supernatant after 24 hours. TNF-α was chosen because an in house TNF-α ELISA assay had already been developed by a colleague (Alexander Edwards, Immunobiology laboratory), whereas the other cytokines were assayed using expensive commercial kits. rMRP-14 induced the expression of TNF-α by RAW 264.7 cells at similar levels to the LPS control (Figure 3.8 D). rMRP-14 did not induce the expression of TNF-α by WEHI3 or P388 cells (data not shown).
Figure 3.7 MIP-2 expression in air pouch tissue after rMRP-14 injection
Immunohistochemistry of air pouch tissue, 1 hour after injection of 50 μg rMRP-14. Sections were stained with A) MIP-2, or B) control goat antisera. This immunohistochemistry was performed by Philippa Munson at University College London. Data representative of 2 experiments.
Figure 3.8 Macrophages respond to rMRP-14 in vivo and in vitro.
A)-C) Immunohistochemistry of air pouch tissue, 1 hour after injection of 50 μg rMRP-14. Sections were stained with A) F4/80; to identify macrophages, B) 7/4; to identify myeloid cells or C) control rat antisera. This immunohistochemistry was performed by George Elia at Cancer Research UK. Data representative of 2 experiments.
D) TNF-α production by RAW 264.7 cells incubated in vitro with rMRP-14 (50 μg/ml), LPS (1 μg/ml) or medium alone. Data are mean ± SD. Data representative of 2 experiments.
3.2.7 Specificity of the response to rMRP-14 in the air pouch

Concomitant with experiments to further understand the mechanism of action of rMRP-14, a number of control experiments were performed. To demonstrate the specificity of the response to rMRP-14 in the air pouch, antibody-blocking experiments were performed. It was hoped that antibody binding to rMRP-14 would block its interaction with its unknown receptor on target cells, and thereby inhibit cytokine production and subsequent leukocyte recruitment. In one experiment, rat mAb 2B10, which recognises an unknown epitope of murine MRP-14, or an isotype IgG2a control antibody, were injected along with rMRP-14 into the air pouch. MAb 2B10 was used in a two-fold molar excess. MAb 2B10 did not block the recruitment of leukocytes by rMRP-14 (Figure 3.9 A), and this experiment was inconclusive. A similar antibody-blocking experiment was performed using Ab NH9, a rabbit polyclonal antibody that recognises murine MRP-14. Unfortunately NH9 also did not block leukocyte recruitment into the air pouch (data not shown) and this experiment was also inconclusive.

Endotoxins were originally identified as bacterial toxins that were resistant to heat inactivation (Rietschel and Brade, 1992). To demonstrate that endotoxin contamination of rMRP-14 was not responsible for its effects in vivo, rMPR-14 and an LPS control were heat inactivated, and their ability to recruit leukocytes into the air pouch was compared with non-boiled samples.
Figure 3.9 The effect of rMRP-14 in vitro may be due to endotoxin contamination
(A-C) Leukocyte migration into the air pouch 6 hours after stimulus injection.
(A) Leukocyte migration induced by rMRP-14 cannot be blocked with mAb 2B10. A buffer control or rMRP-14 alone (50 μg) or in combination with 0.3 mg mAb 2B10 or an isotype control antibody were injected into the air pouch.
(B) Reduced leukocyte migration in response to heat inactivated rMRP-14 and LPS compared to non-boiled samples. Samples of rMRP-14 (50 μg) or LPS (10 ng) were inactivated by boiling at 100 °C for 15 minutes. Boiled and non-boiled samples were injected into the air pouch.
(C) Absence of leukocyte migration in response to LPS and rMRP-14 in LPS insensitive C3H-HeJ mice, but not LPS sensitive C3H-HeN mice.
It was predicted that heat inactivation would abolish the activity of rMRP-14, but not LPS. However both LPS and rMRP-14 boiled samples showed a ~50% reduction in activity compared to non-boiled samples (Figure 3.9 B). Therefore this experiment was inconclusive.

A member of the Toll receptor family, TLR4, is critically required for LPS signal transduction (Triantafilou and Triantafilou, 2002). The first proof of the involvement of TLR4 came from the analysis of the LPS-unresponsive C3H-HeJ mouse strain. C3H-HeJ mice have a point mutation in the Tlr4 gene that modifies a conserved residue in the cytoplasmic domain, which has a dominant-negative effect on LPS signal transduction (Poltorak et al., 1998). As a final control to rule out endotoxin contamination of rMRP-14, an air pouch experiment was performed using C3H-HeJ mice. The response of C3H-HeJ mice to rMRP-14 was compared C3H-HeN mice. The C3H-HeN strain diverged from the same stock as C3H-HeJ mice but exhibit a vigorous response to LPS. C3H-HeN, but not C3H-HeJ mice, were able to mount an inflammatory response to LPS, and both strains were able to respond to TNF-α (Figure 3.9 C). However, a completely unexpected result was that rMRP-14 did not elicit a leukocyte infiltrate into the air pouch of C3H-HeJ mice, whereas the response of C3H-HeN mice was similar to that of C57BL/6 mice. The conclusion from this experiment is that the effect of rMRP-14 in vivo is likely to be due to endotoxin contamination.
3.2.8 LPS contamination can explain the effect of rMRP-14 in vivo.

The endotoxin levels contaminating 50 µg of rMRP-14 had already been measured, and were found to be ~0.4 ng. To test whether low levels of LPS in the air pouch could reproduce the cytokine expression seen with rMRP-14, LPS was titrated from 10 – 0.01 ng and the cytokines in the air pouch exudate were measured. LPS was able to induce both KC and TNF-α expression in the air pouch exudate, as seen with rMRP-14 (Figure 3.10 A,B). The highest levels of cytokines induced by LPS corresponded to a dose of 1 ng, and matched the levels induced by 50 µg rMRP-14. However, LPS at 0.4 ng, corresponding to the amount of endotoxin contaminating 50 µg rMRP-14, was less effective than rMRP-14 at inducing KC and TNF-α.

The ability of LPS to induce TNF-α by RAW 264.7 cells in vitro was then tested. RAW 264.7 cells were incubated with medium alone, rMRP-14 or a range of concentrations of LPS for 24 hours and the level of TNF-α in the culture medium was assayed. rMRP-14 and LPS induced the expression of TNF-α by RAW 264.7 cells. However, 0.4 ng LPS, corresponding to the amount of endotoxin contaminating 50 µg rMRP-14, was less effective than rMRP-14 at stimulating TNF-α production (Figure 3.10 C).
Figure 3.10 LPS alone induces similar chemokines to rMRP-14 *in vivo* and can stimulate production of chemokines by RAW cells

rMRP-14 (50 μg), LPS or buffer alone were injected into the air pouch. 1 hour after stimulus injection air pouch exudate was collected and the levels of KC (A) and TNF-α (B) were measured by ELISA. Data are mean ± SEM. Representative of n=2

(C) Low levels of LPS stimulate TNF-α production by RAW 264.7 cells. RAW 264.7 cells were incubated with rMRP-14 (50 μg/ml), LPS or buffer alone. After 24 hours the culture supernatant was collected and the level of TNF-α was measured by ELISA. Data are mean ± SD. Representative of 2 experiments
3.3 Discussion

3.3.1 Expression and purification of rMRP-14

rMRP-14 was expressed as a His-tagged protein in *E. coli* and successfully purified from the bacterial cytosol using a His-trap column to specifically bind the tagged protein, followed by a Hydroxyapatite column to purify correctly folded Ca$^{2+}$ binding protein. It is possible to remove the histidine tag from recombinant proteins by proteolysis using thrombin. However, previous attempts to do this resulted in thrombin contamination of rMRP-14 (May, 1999). This was undesirable, as thrombin is a potent inflammatory mediator. Importantly, the biological activity of rMRP-14 in the air pouch was not impaired by the presence of the histidine tag, therefore it was decided not to remove it. As endotoxin is a potent inflammatory mediator, every attempt to remove it from rMRP-14 was made, using detergent extraction or KuttsuClean protocols. Contaminating levels of endotoxin were measured using the sensitive Limulus Amoeocyte Lysate Assay, and found to be 0.4 ng or approximately $10^{-14}$ mol per 50 µg rMRP-14, which initially was believed to be biologically insignificant.

3.3.2 rMRP-14 does not cause myeloid cell activation

Chemoattractants can be classified into two groups (Haines et al., 1993). Firstly there are classical chemoattractants such as fMLP and IL-8, which stimulate myeloid cell migration and activation, with mobilisation of intracellular Ca$^{2+}$ and activation of the
Chapter 3: Effect of rMRP-14 in vitro and in vivo

respiratory burst. Secondly, there are “non-classical” chemoattractants such as TGF-β1 and murine MRP-8 (CP-10) that only influence cell shape change and movement.

To investigate the mechanism by which rMRP-14 caused leukocyte migration into the air pouch, the ability of rMRP-14 to cause chemotaxis and activation of myeloid cells in vitro was tested. rMRP-14 did not directly cause chemotaxis of myeloid cells in the Transwell assay. Moreover, rMRP-14 did not cause myeloid cell activation as demonstrated by calcium mobilisation and respiratory burst assays.

A number of S100 proteins have been reported to be directly chemotactic for a number of cell types in vitro (Donato, 2001). Murine MRP-8 and human S100A12 have been shown to be chemotactic both in vitro and in vivo for myeloid cells (Hofmann et al., 1999; Passey et al., 1999b; Yang et al., 2001). Therefore, it was suprising that rMRP-14 was not able to directly cause myeloid cells chemotaxis, and suggested that MRP-14 may have a different mode of action to the other chemotactic S100 proteins described so far. However, preliminary attempts to demonstrate the chemotactic activity of murine MRP-8 in vitro, as previously reported (Lackmann et al., 1992; Lackmann et al., 1993), were unsuccessful.

3.3.3 rMRP-14 causes leukocyte emigration via an indirect mechanism

As in vitro experiments can never fully reproduce the situation in vivo, the mechanism of rMRP-14 induced leukocyte influx into the air pouch was investigated in vivo. rMRP-14 caused a rapid, transient increase the level of cytokines in air pouch fluid, which preceded the leukocyte infiltrate. The levels of TNF-α, KC, MIP-2, MCP-1 and MIP-1α in air pouch exudate peaked at 1 hour, and decreased to near
basal levels by 6 hours, the time corresponding to the peak leukocyte recruitment. The temporal production of TNF-α, KC and MIP-2 was almost identical, whereas the levels of MIP-1α and MCP-1 were more sustained. How these observations relate to the respective biological functions of these cytokines in vivo is unclear. The relative importance of the different cytokines in the recruitment of different leukocyte subsets into the air pouch could be further investigated by testing the effect of specific neutralising antibodies on leukocyte extravasation.

The transient production of cytokines preceding leukocyte recruitment has been reported in several inflammatory models (Ajuebor et al., 1999; Tessier et al., 1997; Tessier et al., 1998). For example, TNF-α in the air pouch model caused a rapid and transient increase in the exudate levels of MIP-2, MIP-1α, and JE, which peaked at 1 hour and decreased considerably by 2 – 4 hours, the time corresponding to the peak leukocyte infiltrate. The transient induction of cytokines can be attributed to their rapid clearance from the inflammatory site, by degradation or diffusion, and to the down-regulation of their synthesis, which has been shown to be regulated at the level of transcription (Tessier et al., 1997).

3.3.4 Macrophages are a major cell type responding to rMRP-14 in vivo

There is increasing evidence that leukocytes recruited to inflammatory sites are an important source of cytokines. For example, in vivo studies have demonstrated that neutrophils can synthesize a variety of cytokines including MIP-2, KC, MIP-1α and MIP-1β (Scapini et al., 2000). However, as there were very few recruited leukocytes at the time corresponding to maximal cytokine production by rMRP-14, it was likely
that the majority of cytokines in air pouch exudate were produced by the resident air pouch cells. The importance of resident air pouch cells in the production of cytokines in response to inflammatory stimuli is well accepted (Garcia-Ramallo et al., 2002; Terkeltaub et al., 1998). Following rMRP-14 injection, MIP-2 was produced by cells within, and beneath, the epithelial lining of the air pouch. The majority of these cells were identified as macrophages based on their staining with mAb F4/80. Other studies have shown that in addition to macrophages, other cells including fibroblasts, smooth muscle cells, and epithelial cells, may synthesize cytokines in response to inflammatory stimuli (Ajuebor et al., 1999; Garcia-Ramallo et al., 2002; Terkeltaub et al., 1998).

The induction of cytokines by rMRP-14 was reproduced in an in vitro assay. RAW 264.7 cells produced TNF-α in response to rMRP-14. It was thought that this assay would be useful to test new batches of rMRP-14 for functional activity and serve as a model to further investigate the mechanism of rMRP-14 in vitro.

3.3.5 The effect of rMRP-14 is likely to be due to endotoxin contamination

Various control experiments were performed to exclude the possibility that endotoxin contamination of rMRP-14 was responsible for its effect in vivo. As antibody blocking and heat inactivation experiments were inconclusive, a control air pouch experiment was performed using LPS insensitive C3H-HeJ mice. An unexpected result was that rMRP-14 did not cause a leukocyte infiltrate in these mice. Moreover, LPS alone was able to induce a similar panel of cytokines to rMRP-14. LPS at a concentration corresponding to amount to the level of endotoxin contaminating 50 μg rMRP-14 was less potent at inducing cytokines than rMRP-14.
These differences in potencies may be due to several factors. Firstly, the purified LPS was from a different bacterial strain to that used to express rMRP-14. Different bacterial strains can have different potencies of LPS (Henderson and Wilson, 1996). Secondly, LPS contaminating rMRP-14 was likely to be present in macromolecular complexes containing protein, LPS, and possibly phospholipid and nucleic acid as well. Such complexes are likely to affect the potency of the LPS preparation (Henderson and Wilson, 1996).

It is widely believed that LPS is resistant to heat inactivation, therefore it is unexpected that the biological activity of endotoxin contaminated rMRP-14 is sensitive to heat inactivation. However, a recent study has demonstrated that the sensitivity of LPS to heat inactivation is concentration dependent. At low LPS concentrations, similar to those contaminating rMRP-14, LPS is heat sensitive (Gao and Tsan, 2003). In summary, the effect of rMRP-14 in vivo is likely to be due to endotoxin contamination.

3.3.6 In vivo studies of LPS

LPS is known to be a potent inflammatory mediator, but the effect of LPS in the air pouch model is not well characterised. In other inflammatory models, LPS has been shown to be a potent stimulus for neutrophil emigration. Studies in rabbits investigating leukocyte emigration, in response to intradermal injection of LPS found that neutrophil emigration was detected at just 12 pg LPS/injection site (Cybulsky et al., 1988). The importance of tissue macrophages in the response to LPS in vivo has been previously reported (Ajuebor et al., 1999; Harmsen and Havell, 1990). The selective depletion of macrophages demonstrated that they play a key role in the
Chapter 3: Effect of rMRP-14 in vitro and in vivo

recruitment of neutrophils into the peritoneum, in response to LPS (Ajuebor et al., 1999).

The kinetics of leukocyte recruitment into the air pouch in response to rMRP-14 are consistent with previously published inflammatory models using LPS. The kinetics of neutrophil emigration towards intradermal LPS were rapid and transient, peaking at 2 hours post injection and decreasing to baseline levels by 8 hours (Cybulsky et al., 1988). In a model of peritonitis, LPS injection caused an influx of neutrophils that peaked between 6-16 hours and decreased to near basal levels by 48 hours (Ajuebor et al., 1999). These results suggest that different tissue environments influence the kinetics of leukocyte recruitment in response to LPS, but in general, the response is delayed and transient.

The range of cytokines produced in response to LPS has been previously reported in a model of peritonitis. LPS caused the expression of both KC and MCP-1 (Ajuebor et al., 1999), which were also induced by rMRP-14 in the air pouch. TNF-α is known to be a major mediator released by tissue macrophages in response to LPS (Rietschel and Brade, 1992) and TNF-α alone, can mimic several of the responses attributed to endotoxin (Rietschel and Brade, 1992). The induction of chemokine expression by TNF-α in the air pouch has been previously reported (Tessier et al., 1997). Injection of TNF-α had a similar effect to rMRP-14 in the air pouch, and caused the expression of KC, MIP-2 and MIP-1α in the air pouch exudate.

The finding that rMRP-14 can stimulate TNF-α expression by RAW 264.7 cells but not WEHI3 and P388 cells is consistent with the variable responses of these cell lines to LPS. It has been reported that while LPS triggers a priming signal in
P388 cells, RAW 264.7 cells are fully activated to release inflammatory mediators (Barbour et al., 1998). These differences have been suggested to be due to differences in signalling events elicited by LPS that are related to the mechanisms and/or extent of their immortalisation of these cell lines.

**3.3.7 Could rMRP-14 be a ligand for TLR4?**

The possibility exists that rMRP-14 itself, and not LPS, is activating cells through TLR-4. This is unlikely as the level of endotoxin containing rMRP-14 has potent effects *in vivo*. This suggestion could be tested an endotoxin free preparation of MRP-14. One idea is to produce MRP-14 using a different expression system, e.g. a eukaryotic cell line, but great care must be taken at all stages of purification, as the protein could still acquire endotoxin contamination from glassware and many commercial chemicals and solutions. To conclusively demonstrate that the activity of rMRP-14 is due to endotoxin contamination, the activity should be shown to be insensitive to protease digestion, and be blocked by polymyxin B or neutralising antibodies to CD14.

Recently endotoxin contamination has been shown to mediate the effects of a commercially available recombinant protein, Hsp70 (Gao and Tsan, 2003). Low levels of endotoxin present in Hsp70, similar to those found in rMRP-14, were responsible for its ability to induce TNF-α expression by macrophages (Gao and Tsan, 2003). Clearly, the potent biological effects of endotoxin contaminating recombinant proteins need to be more widely publicised.
3.3.8 Recombinant human MRP-14 is also a potent chemoattractant in vivo

Recent work by Ryckman and co-workers reports the characterisation of the proinflammatory properties of recombinant human (rh) MRP-14, rhMRP-8 and rhMRP-8/14 (Ryckman et al., 2003). These proteins were expressed using a similar *E. coli* expression system as used in this chapter and their proinflammatory properties characterised using the same air pouch model. 10 μg of rhMRP-8, rhMRP-14 or the heterodimer all induced a transient accumulation of neutrophils and monocytes in the air pouch model using CD-1 mice, with identical kinetics to those reported for rMRP-14 in this chapter. Neutrophil migration was maximal at 6 hours and fell to background levels by 24 hours. However, the rh proteins also produced an inflammatory infiltrate in the air pouch model when LPS insensitive C3H-HeJ mice were used. These findings conflict with those reported in this chapter, despite the similarities between the experimental protocols.

Ryckman and co-workers also find rhMRP-8, rhMRP-14 and the heterodimer to be directly chemotactic and chemokinetic for human neutrophils *in vitro*, inducing migration within 30 minutes. However, neutrophil migration was only stimulated 2-fold over baseline and no positive control, such as IL-8 is included. They do not comment on the discrepancy between the kinetics of MRP stimulated neutrophil migration *in vitro* and *in vivo*. Interestingly, mAbs to rhMRP-8 and rhMRP-14 blocked neutrophil migration *in vitro*, but the ability of these mAbs to block neutrophil migration *in vivo* was not tested. The possibility of endotoxin contamination was excluded by showing that the chemotactic activity of the rh proteins could be heat inactivated. It is unclear why fMLP and not LPS was used as a heat resistant control. In summary, the reported proinflammatory effects of rhMRP-8...
and rhMRP-14 *in vivo* using CD1 mice are very similar to those reported for rMRP-14 in this chapter. However, there are major differences in the abilities of the recombinant human and murine proteins to cause neutrophil chemotaxis *in vitro* and *in vivo*, using C3H-HeJ mice. The reasons for these discrepancies are unclear.
CHAPTER 4

Initial characterisation of MRP-14\(^{-/}\) mice

4.1 Introduction

Numerous intracellular functions have been proposed for S100 proteins based on \textit{in vitro} studies (Donato, 2001). However, the physiological functions of S100 proteins have remained largely elusive. Only four S100 protein-deficient mouse models have been reported. MRP-8\(^{-/}\) mice die \textit{in utero} (Passey et al., 1999a), suggesting that MRP-8 has a role at an early stage in development, possibly in fetal-maternal interactions (Passey et al., 1999a). This is in contrast to S100B\(^{-/}\) and S100A1\(^{-/}\) mice that are viable and display no gross abnormal phenotype (Du et al., 2002; Nishiyama et al., 2002; Xiong et al., 2000). S100B\(^{-/}\) astrocyte cultures from 6 day old mice display enhanced Ca\(^{2+}\) transients upon stimulation, indicative of a buffering function of S100B (Xiong et al., 2000). However, the enhanced Ca\(^{2+}\) transients were not observed in mature astrocytes in hippocampal slices \textit{in vivo} (Nishiyama et al., 2002). S100B\(^{-/}\) mice display enhanced synaptic plasticity, suggesting that glial cell derived S100B has an extracellular role in influencing neuronal activity (Nishiyama et al., 2002). S100A1 protein is highly expressed by cardiomyocytes. The cardiac function of S100A1\(^{-/}\) mice is normal under baseline conditions, but has reduced responses after \(\beta\)-adrenergic stimulation that is associated with a reduced Ca\(^{2+}\) sensitivity (Du et al., 2002). These findings are consistent with S100A1 performing specific effector functions, and not simply Ca\(^{2+}\) buffering functions.
To gain insight into the physiological role of MRP-14, Richard May in this laboratory generated MRP-14 deficient mice using an insertional vector strategy. This work was done at Cancer Research UK in collaboration with Ian Rosewell, Mary Ann Jacobs and Stephen Wilson. The MRP-14 gene was disrupted by insertion of the \textit{Lac Z/Neo} cassette into exon 2 immediately following the transcriptional start site (Figure 4.1 A). Correctly targeted 129-derived ES cell clones were injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric mice were then bred with C57BL/6 mice to generate heterozygotes that were a mixture of 129 and C57BL/6 strains. Two independent strains of mice, 2E5 and 2G1, were established. Interbreeding heterozygotes produced MRP-14\(^{-}\) mice that were viable.

The aim of this part of the project was to perform basic characterisation of these mice.
4.2 Results

4.2.1 Animal husbandry

The MRP-14 wild type and mutant alleles were distinguished using a PCR based assay (Figure 4.1). In wild type alleles, primers GFl and GB1 bound to sequences within exon 1 and exon 2 to generate a 300 bp PCR product. In the mutant allele, insertion of the \textit{Lac Z/Neo} cassette near the start of exon 2 prevented synthesis of this fragment. Instead, GB1 in combination with another primer, GNEO, which bound to a 3' region within the cassette generated a 100 bp product in the mutant allele. This PCR technique confirmed both the presence and correct targeting of the \textit{Lac Z/Neo} cassette in MRP-14\textsuperscript{+/+} mice.

Interbreeding of heterozygotes yielded the proportions of 1.0:1.9:1.0 for MRP-14\textsuperscript{+/+}: MRP-14\textsuperscript{+/}: MRP-14\textsuperscript{-/-} mice, approximating the expected 1:2:1 ratio for Mendelian inheritance, indicating that MRP-14 protein is not essential for embryonic development. C57BL is the most widely used of all the inbred mouse strains, and the C57BL/6 substrain alone accounts for 14% of occasions on which an inbred strain is used. Therefore, heterozygous mice were further back-crossed (BX) with C57BL/6 mice, and then this was repeated with their heterozygous offspring and so on, to generate transgenic mice on a pure C57BL/6 background. Over the course of this thesis, mice were bred from BX1 to BX9.
Figure 4.1 Targeted inactivation of the MRP-14 gene

(A) The murine MRP-14 gene consists of 3 exons (grey bars). Disruption of the MRP-14 gene was achieved by insertion of the Lac Z/Neo cassette into exon 2 immediately following the transcriptional start site. BH, BamHI; B, BglII; EL, EcoRI; S, SacI; X, XbaI. Primer binding sites are represented by coloured circles; Green, GF1; Purple, GB1; Red, GNEO.

(B) Ethidium bromide-stained 1.8% agarose gel of PCR products generated from amplified mouse tail DNA.
Chapter 4: Initial characterisation of MRP-14\(^+\) mice

For *in vitro* experiments, sex matched littermates were used. To generate large numbers of mice for *in vivo* experiments, MRP-14\(^{+/+}\) x MRP-14\(^{+/+}\) and MRP-14\(^{+/-}\) x MRP-14\(^{+/-}\) pairings were set up at the highest level of back cross available.

As MRP-14 is highly expressed by human neutrophils and monocytes, it was predicted that MRP-14\(^{-/-}\) mice might have a defect in innate immune function and an increased susceptibility to infection. Therefore, all mice were housed in a full barrier unit, in a specific pathogen free environment. No increase in morbidity and mortality was observed in MRP-14\(^{-/-}\) mice up to 2 years of age.

### 4.2.2 Normal tissues and organs in MRP-14\(^{+/-}\) mice

MRP-14\(^{+/-}\) mice had no detectable tissue or organ abnormalities. Tissues from MRP-14\(^{+/+}\) and MRP-14\(^{+/-}\) mice were examined for MRP-14 expression by immunohistochemistry using mAb 2B10. The following organs were examined: liver, kidney, large and small intestine, skin, thymus, peripheral lymph nodes and spleen. Positive MRP-14 staining was restricted to myeloid cells in MRP-14\(^{+/+}\) mice and was most readily observed in spleen sections (Figure 4.2 A, C). No staining was evident in spleen sections of MRP-14\(^{+/-}\) mice (Figure 4.2 B). The basic organisation of the spleen was similar in MRP-14\(^{+/+}\) and MRP-14\(^{+/-}\) mice and similar numbers of myeloid cells were detected in the red pulp areas (Figure 4.2 E, F).
Figure 4.2 Tissue sections of spleen from MRP-14+/+ and MRP-14−/− mice. Spleen sections showing positive staining of MRP-14 expressing myeloid cells scattered within the red pulp area using mAb 2B10 in MRP-14+/+ (A,C) but not MRP-14−/− mice (B); (C) Higher power magnification of spleen from a MRP-14+/+ mouse showing both moderate (monocyte) and strong (neutrophil) staining of myeloid cells. (D) Staining with an IgG2a isotype control antibody. Myeloid cells were identified using mAb 7/4 in MRP-14+/+ (E) and MRP-14−/− (F) spleens. Immunohistochemistry performed by George Elia at Cancer Research UK. PALS, periarteriolar lymphoid sheath. Scale bar = 500 μm.
4.2.3 Lack of MRP-14 expression in MRP-14<sup>+</sup> monocytes and neutrophils

Close examination of spleen sections from MRP-14<sup>+</sup> mice revealed two populations of cells expressing moderate and high amounts of MRP-14 (Figure 4.2 C). It was likely that these cells corresponded to murine monocytes and neutrophils respectively, similar to the human situation (Edgeworth et al., 1991; Hessian et al., 1993). As the murine monocyte is difficult to identify, the expression of MRP-14 by murine monocytes has not been previously characterised. A new phenotyping technique, using a combination of mAbs 7/4 and Gr-1 to identify murine neutrophils (7/4<sup>+</sup>, Gr-1<sup>+</sup>) and monocytes (7/4<sup>+</sup>, Gr-1<sup>inv</sup>) (Henderson et al., 2003) allowed MRP-14 expression by murine myeloid cells to be investigated.

To test whether MRP-14 is expressed by monocytes, bone marrow myeloid cells were stained with mAbs 7/4 and Gr-1, and neutrophil and monocyte populations were isolated using a cell sorter. Western blot analysis of neutrophil and monocyte cell lysates showed that MRP-14 was expressed at high levels in neutrophils and to a lesser extent in monocytes (Figure 4.3 A). The presence of MRP-14 in monocytes was confirmed by an intracellular staining protocol. Bone marrow myeloid cells were identified by staining with mAbs 7/4 and Gr-1, fixed and permeabilised, and intracellular MRP-14 expression was detected by staining with mAb 2B10. MRP-14<sup>+/+</sup> and MRP-14<sup>+</sup> mice express the epitope of mAb 7/4 at similar high levels (Figure 4.3 B).
Figure 4.3 MRP-14 protein is absent in MRP-14\(^{+}\) monocytes and neutrophils

(A) Western blot analysis of MRP-14 expression in MRP-14\(^{+}\) bone marrow neutrophil (Neut) and monocyte (Mono) lysate. Myeloid cells were stained with mAbs Gr-1 and 7/4 and isolated using a cell sorter (B) MRP-14\(^{+}\) and MRP-14\(^{-}\) bone marrow myeloid cells were identified by flow cytometry by staining cells with mAb 7/4. (C) The mAb 7/4\(^{+}\) myeloid cells (R1) from MRP-14\(^{+}\) and MRP-14\(^{-}\) mice were intracellularly stained with either mAb 2B10 specific for MRP-14 (shaded histogram) or an IgG2a isotype control mAb (open histogram). (D) In MRP-14\(^{+}\) mice two positive populations can be seen (R2,R3) which are absent in MRP-14\(^{-}\) myeloid cells. These populations correspond to monocytes (R2) and neutrophils (R3) based on their differential staining with mAb Gr-1 (D) and their Forward scatter/Side scatter profiles (E). Inset numbers represent the geometric mean fluorescence of the Gr-1 positive populations. Data representative of 3 experiments.
In MRP-14\(^{+/+}\) mice, the mAb 7/4\(^{+}\) population is composed of two subsets of cells (R2 and R3) expressing different levels of MRP-14 (Figure 4.3 C). These populations correspond to monocytes (R2, low MRP-14) and neutrophils (R3, high MRP-14) respectively, as demonstrated by their differential staining with mAb Gr-1 (Figure 4.3 D) and forward/side scatter profiles (Figure 4.3 E). In MRP-14\(^{-/-}\) mice, monocytes and neutrophils failed to stain positively with mAb 2B10 (Figure 4.3 C). In summary, MRP-14 is expressed by monocytes and neutrophils in MRP-14\(^{+/+}\) but not MRP-14\(^{-/-}\) mice.

4.2.4 Absence of MRP-8 protein but not MRP-8 mRNA in MRP-14\(^{-/-}\) neutrophils

MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) bone marrow cells were analysed for expression of MRP-14 and MRP-8 mRNA. RNase protection assays demonstrated the absence of MRP-14 mRNA in MRP-14\(^{-/-}\) mice, and, as expected, normal levels of MRP-8 mRNA (Christoffer Gebhardt, personal communication). The expression of a truncated form of MRP-14 was tested by RT-PCR, using primers to amplify exon 3 that proceeds the \textit{Lac Z/Neo} cassette insertion site. MRP-14 exon 3 mRNA was detected in MRP-14\(^{+/+}\) but not MRP-14\(^{-/-}\) mice (Eileen McNeill, personal communication).

The level of MRP-14 and MRP-8 proteins in MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) bone marrow cell lysates was tested by Western blotting of 2D gels, using Abs 2B10 and NH9 respectively. In the MRP-14\(^{+/+}\) lysate, mAb 2B10 detected a single protein spot (Figure 4.4 B).
Figure 4.4 MRP-14 and MRP-8 expression in MRP-14⁺⁻ bone marrow
Three 2D gels of MRP-14⁺⁻ bone marrow cell lysate were run in parallel. Following separation using an immobilised pH gradient with a non-linear pH range of 3 - 10, proteins were transferred on to a 17.5% polyacrylamide gel, which was then stained with colloidal coomassie stain (A) or transferred onto nitrocellulose membrane (B,C). The transfer was confirmed by staining membranes with Ponceau Red, three strong bands of protein were visualised on each (marked with arrows), then MRP-14 (B) and MRP-8 (C) were detected by Western blotting using mAb 2B10 and rabbit polyclonal NH9 respectively. Data representative of 2 experiments.
Chapter 4: Initial characterisation of MRP-14/− mice

Ab NH9 recognised one major protein spot and two minor slightly more acidic spots in the MRP-14+/+ lysate, one of the same molecular weight and one slightly smaller (Figure 4.4 C). MRP-14 protein was undetectable in MRP-14/− bone marrow lysate using mAb 2B10 (Figure 4.5 B). The mAb 2B10 staining conditions were such that MRP-14 protein was not detectable in the MRP-14/− cells at >256 times the detectable loading of the MRP-14+/+ cells (Figure 4.6 A). Surprisingly, no MRP-8 protein was detected in MRP-14/− cells using Ab NH9 (Figure 4.5 C). The Ab NH9 staining conditions were such that MRP-8 protein was not detectable in MRP-14/− cells at >64 times the detectable loading of MRP-14+/+ lysates (Figure 4.6 B). In summary, MRP-8 mRNA but not protein is detectable in MRP-14/− bone marrow.

4.2.5 Lack of compensatory changes in the levels of other proteins in MRP-14/− myeloid cells

To test for proteins whose expression was altered to compensate for the loss of MRP-14 and MRP-8, MRP-14+/+ and MRP-14/− bone marrow lysates were compared by 2D gel analysis (Figure 4.7). Following isoelectric focussing, proteins were separated on a 17.5% acrylamide gel and visualised with coomassie blue. The high percentage acrylamide gel gave good separation of low molecular weight proteins, including other S100 proteins, which could be upregulated in MRP-14/− mice. Statistical analysis was used to compare protein spots of two groups of four MRP-14+/+ and four MRP-14/− lysates. The only proteins that were significantly altered out of the 175 protein spots examined corresponded to MRP-8 and MRP-14, which had been previously identified by Western blotting.
Figure 4.5 MRP-14 and MRP-8 expression in MRP-14⁺ bone marrow

Three 2D gels of MRP-14⁺ bone marrow cell lysate were run in parallel. Following separation using an immobilised pH gradient with a non linear pH range of 3 - 10, proteins were transferred on to a 17.5% polyacrylamide gel, which was then stained with colloidal coomassie stain (A) or transferred onto nitrocellulose membrane (B,C). The transfer was confirmed by staining membranes with Ponceau Red, three strong bands of protein were visualised on each (marked with arrows), then MRP-14 (B) and MRP-8 (C) were detected by Western blotting using mAb 2B10 and rabbit polyclonal NH9 respectively. Data representative of 2 experiments.
Figure 4.6 Sensitivity of MRP-8 and MRP-14 detection by Western blotting
MRP-14+/+ bone marrow cell lysate at a series of dilutions and undiluted MRP-14−/− lysate was separated on a 17.5% polyacrylamide gel, transferred onto nitrocellulose membrane, then MRP-14 (A) and MRP-8 (B) were detected by Western blotting using mAb 2B10 and rabbit polyclonal NH9 respectively. M, Molecular weight markers.
Figure 4.7 2D gel analysis of MRP-14^{+/+} and MRP-14^{-/-} bone marrow lysates. Proteins in bone marrow lysates from MRP-14^{+/+} (A) and MRP-14^{-/-} (B) mice were separated using an immobilised pH gradient with a linear pH range of 3 - 10. Proteins were then transferred on to a 17.5% polyacrylamide gel, which was then stained with colloidal coomassie. The positions of MRP-8 and MRP-14 are highlighted with a dashed line.
The lack of compensatory changes in other S100 proteins was also confirmed using RT-PCR. No alterations in the expression of S100A1 (Du et al., 2002) or S100A4 (Grigorian et al., 1994), that are generally expressed by leukocytes, was detected (Eileen McNeill, personal communication).

4.2.6 Expression of MRP-8 mRNA during embryonic development in MRP-14<sup>−/−</sup> mice.

MRP-8 has been reported to be expressed between day 6.5 and 8.5 days post coitum (d.p.c.) of embryonic development and lack of MRP-8 has been shown to cause embryonic lethality by 9.5 d.p.c. in MRP-8<sup>−/−</sup> mice (Passey et al., 1999a). The absence of MRP-8 protein in the myeloid cells of MRP-14<sup>−/−</sup> mice raised the issue as to how these mice could be viable. To investigate the expression of MRP-8 during embryonic development, <i>in situ</i> hybridisation was performed on MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> sections at 6.5 and 7.5 d.p.c.. The <i>in situ</i> hybridisation was performed by Rosemary Jeffery at Cancer Research UK. In agreement with Passey <i>et al.</i>, there was extensive MRP-8 expression at 6.5 d.p.c. (data not shown) and 7.5 d.p.c. in MRP-14<sup>+/+</sup> sections (Fig. 4.8 A,C). The expression of MRP-14 mRNA was also examined. MRP-14 was detected in MRP-14<sup>+/+</sup> mice in a small area of decidual tissue (Figure 4.8 B,D), which only partially overlapped with the region of MRP-8 expression. In MRP-14<sup>−/−</sup> sections, MRP-8 mRNA had the same localisation pattern as in the MRP-14<sup>+/+</sup> sections, but the expression levels were substantially diminished (Fig. 4.9 A,C). As expected, MRP-14 mRNA was not detected in MRP-14<sup>−/−</sup> sections (Figure 4.9 B,D).
Figure 4.8 Expression of MRP-8 and MRP-14 mRNA during embryonic development in MRP-14<sup>−/−</sup> mice at 7.5 d.p.c.

In each longitudinal section, the remnant of the uterine lumen (U) is evident at the top of the section with developing embryo visible below. Bright and dark field images of in situ hybridisations showing MRP-8 (A,C) and MRP-14 (B,D; see arrows) mRNA expression. (E) Hybridisation with a β-actin probe was used as a positive control for embryonic and maternal expression. Images are serial sections and are representative of 6 determinations. Scale bar = 0.5 mm. E; embryo, M; maternal tissue; U; remnant of uterine lumen. These in situ hybridisations were performed by Rosemary Jeffery at Cancer Research UK.

148
Figure 4.9 Expression of MRP-8 but not MRP-14 mRNA during embryonic development in MRP-14<sup>−/−</sup> mice at 7.5 d.p.c.

In each longitudinal section, the remnant of the uterine lumen (U) is evident at the top of the section with developing embryo (E) visible below. Bright and dark field images of in situ hybridisations showing MRP-8 (A,C; see arrows) and MRP-14 (B,D) mRNA expression. (E) Hybridisation with a β-actin probe was used as a positive control for embryonic and maternal expression. Images are serial sections and are representative of 6 determinations. Scale bar = 0.5 mm. E; embryo, M; maternal tissue; U; remnant of uterine lumen. These in situ hybridisations were performed by Rosemary Jeffery at Cancer Research UK.
Chapter 4: Initial characterisation of MRP-14<sup>+/+</sup> mice

As a positive control, β-actin showed a wide distribution of staining in MRP-14<sup>+/+</sup> and MRP-14<sup>+/−</sup> embryonic and maternal tissue (Figures 4.8 E, 4.9 E).

4.2.7 Expression of MRP-8 mRNA by maternal decidual cells

Passey et al., report that the cells expressing MRP-8 mRNA are embryonic trophoblasts that have migrated from the ectoplacental cone (e.p.c.) into the surrounding maternal tissue (Passey et al., 1999a). To verify their claim that MRP-8 expression is fetal rather than maternal, a green fluorescent chromosome paint was used to detect Y-chromosomes. This staining was performed by Rosemay Jeffery at Cancer Research UK. The Y-chromosome probe colocalised with nuclei in male fetal cells but not maternal cells. There was no staining of fetal cells within the maternal decidual tissue (Figure 4.10 A) and the region of MRP-8 mRNA expression comprised only maternal tissue (illustrated in Figure 4.10 B).

To test if MRP-8 and MRP-14 proteins were expressed in decidual tissue immunohistochemistry was performed on MRP-14<sup>+/+</sup> sections. Positive staining for MRP-8 and MRP-14 proteins was only detectable in occasional myeloid cells scattered within the decidual tissue (Figure 4.11 and data not shown).
4.10 Fluorescent Y chromosome labelling of male embryonic tissue

(A) Longitudinal section of a male MRP-14+/+ embryo within maternal decidual tissue at 7.5 d.p.c.. Nuclei of maternal and fetal cells were labelled with DAPI (blue). Y-chromosomes in fetal nuclei were identified using a FITC-labelled Y-chromosome paint (green spots). The boundary of maternal (M) and embryonic (E) tissue is indicated with a dashed line. e.p.c.; fetal ectoplacental cone. Scale bar = 0.1 mm. (B) Diagram illustrating that the area of MRP-8 expression (pale blue stripes) is localised to maternal tissue (M; blue) and not embryonic tissue (E; white). Regions shown in (A) are indicated with boxes. This staining was performed by Rosemary Jeffery at Cancer Research UK.
4.2.8 Expression of MRP-8 mRNA by placental tissue

To test whether MRP-8 and MRP-14 mRNA was expressed by extra-embryonic tissue, *in situ* hybridisation was performed on MRP-14*+/−* placental tissue at 10.5, 12 and 14.5 d.p.c.. MRP-8 mRNA was widely expressed by cells within the labyrinth of the placenta at 10.5 but not 12.5 or 14.5 d.p.c. (Figures 4.12, 4.13 A,B and data not shown). MRP-14 mRNA was undetectable in placental tissue (Figure 4.13, C). As a positive control, β-actin showed a wide distribution of staining in placental tissue (Figure 4.13 D).

To test if MRP-8 protein was expressed in the placenta, immunohistochemistry was performed on MRP-14*+/−* sections. Positive staining for MRP-8 was only detectable in very occasional myeloid cells scattered within the decidual tissue (Figure 4.13).
Figure 4.11 Expression of MRP-8 protein during embryonic development in MRP-14+/+ mice at 7.5 d.p.c.
(A) Section of maternal decidual tissue immediately above the embryo showing MRP-8 expression in neutrophils (arrow heads). (B) Serial section showing a similar region stained with an isotype control antibody. Sections are representative of n=3. E; embryo, M; maternal tissue. Scale bar = 0.2 mm. This immunohistochemistry was performed by George Elia at Cancer Research UK.
Figure 4.12 Expression of MRP-8 mRNA in the labyrinth of the placenta at 10.5 d.p.c.

(A) Haematoxylin and eosin stained placental tissue.
(B) *in situ* hybridisation of placental tissue showing MRP-8 mRNA expression. Scale bar = 0.5 mm. Sections are representative of *n*=2. This *in situ* hybridisation was performed by Rosemary Jeffery at Cancer Research UK.
Figure 4.13 Expression of MRP-8 but not MRP-14 mRNA in the placenta at 10.5 d.p.c.
Bright (A) and dark field (B-D) images of *in situ* hybridisations showing MRP-8 (A,B) and MRP-14 (C) mRNA expression. Hybridisation with a β-actin probe was used as a positive control (D). (A-D) Scale bar = 0.2 mm. *In situ* hybridisations were performed by Rosemary Jeffery at Cancer Research UK.

(E) Immunohistochemistry of placental tissue showing MRP-8 protein expression in scattered neutrophils (arrow head). (F) Staining of a similar section with an isotype control antibody. (E-F) Scale bar = 0.1 mm. This immunohistochemistry was performed by George Elia at Cancer Research UK.
4.2.9 Normal numbers and morphology of MRP-14<sup>−</sup> cells

As murine MRP-8/14 is expressed by myeloid progenitors and myeloid cells (Lagasse and Weissman, 1992), the development of the myeloid lineage was examined in MRP-14<sup>−/−</sup> mice. The numbers of myeloid cells in the bone marrow, blood and spleen of MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> mice were counted (Figure 4.14). No difference in the numbers of myeloid cells was seen in MRP-14<sup>−/−</sup> compared to MRP-14<sup>+/+</sup> mice. The numbers of circulating CD4 and CD8 T lymphocytes, B lymphocytes and NK cells were also unaffected (Fig. 4.14).

As MRP-8 and MRP-14 are very abundant cytosolic proteins, the ultrastructure of myeloid cells was examined by transmission electron microscopy, to test whether loss of these proteins had impact on the cellular architecture. This microscopy was performed by Carol Upton at Cancer Research UK. There was no difference in the organelles of either peripheral blood or bone marrow neutrophils or monocytes (Figures 4.15, 4.16 and data not shown).
4.14 Normal leukocyte populations in MRP-14\(^{+/+}\) mice
Leukocytes were isolated from bone marrow (A), blood (B), spleen (C) and lymph nodes (D) from MRP-14\(^{+/+}\) (green bars) and MRP-14\(^{-/-}\) (red bars) mice. Leukocytes were identified and counted by flow cytometry as described in materials and methods. N, Neutrophils; M, Monocytes; CD4/8\(^{+}\), CD4/8\(^{+}\) T lymphocytes; B, B lymphocytes; NK, Natural killer cells. Data representative of 3 experiments.
4.15 Neutrophil architecture is unaltered in MRP-14+ mice

Transmission electron micrographs of peripheral blood neutrophils from MRP-14+/+ (A) and MRP-14+/ (B) mice. Neutrophils were labelled with mAbs 7/4 and Gr-1 and isolated using a cell sorter.

Scale bar represents 500 nm. This electron microscopy was performed by Carol Upton at Cancer Research UK.
4.16 Monocyte architecture is unaltered in MRP-14⁻/⁻ mice
Transmission electron micrographs of peripheral blood monocytes from MRP-14⁺/+ (A) and MRP-14⁻/⁻ (B) mice. Monocytes were labelled with mAbs 7/4 and Gr-1 and isolated using a cell sorter. Scale bar represents 500 nm. This electron microscopy was performed by Carol Upton at Cancer Research UK.
Chapter 4: Initial characterisation of MRP-14\(^{+/−}\) mice

The density of neutrophils from MRP-14\(^{+/+}\) and MRP-14\(^{−/−}\) mice was measured by Percoll\(^\text{TM}\) density gradient centrifugation. 80% of the total neutrophils were recovered from a single band with a reduced mean buoyant density in the MRP-14\(^{−/−}\) samples (1.0811 g/ml) in comparison with the MRP-14\(^{+/+}\) samples (1.0836 g/ml) (Eileen McNeill, personal communication). The reduced density of MRP-14\(^{−/−}\) myeloid cells had no impact on the flow cytometric scatter properties (Margaret Mathies, personal communication). Thus there was a statistically significant difference (p < 0.001) in density between MRP-14\(^{+/+}\) and MRP-14\(^{−/−}\) neutrophils of 2.5 mg/ml.

To examine the activation state and maturity of myeloid cells, the expression of adhesion receptors by MRP-14\(^{+/+}\) and MRP-14\(^{−/−}\) cells was tested. The levels of L-selectin, LFA-1, Mac-1, \(β_1\) integrins and \(α_4\) integrins were similar on MRP-14\(^{+/+}\) and MRP-14\(^{−/−}\) myeloid cells (Figure 4.17).
4.17 Normal adhesion receptor expression on MRP-14<sup>+</sup> myeloid cells

MRP-14<sup>++/+</sup> (green histogram) and MRP-14<sup>+-</sup> (red histogram) peripheral blood leukocytes were isolated using a Histopaque gradient and neutrophils and monocytes were identified by staining cells with mAbs 7/4 and Gr-1. The expression of adhesion receptors was determined by colabelling cells with a panel of antibodies as described in materials and methods. Inset numbers are the median fluorescences of MRP-14<sup>++/+</sup> (green) and MRP-14<sup>+-</sup> (red) histograms.
4.3 Discussion

4.3.1 Animal husbandry

The 129 mouse strain is not a commonly studied inbred mouse strain, but has been increasingly used in the production of transgenic animals due to the availability of several lines of 129 embryonic stem cells. MRP-14\(^{+/-}\) mice were produced using 129 embryonic stem cells and were subsequently backcrossed onto a C57BL background, which is the most widely used of all inbred strains. Backcrossing mice is a slow process and requires multiple rounds of genotyping at each backcross level. There are now an increasing variety of embryonic stem cell strains, including C57BL/6, which were not available at the outset of this project, which would have removed the need to backcross.

Comprehensive phenotypic information on the various inbred strains of mice has recently started to be collated in the Mouse Phenome project. At the outset of characterising the MRP-14\(^{+/-}\) mice, I was not aware of the phenotypic differences between the different mouse strains, and between male and female mice of the same strain. For example C57BL/6 have less than the average percentage of peripheral neutrophils compared to other inbred strains and C57BL/6 female mice have 2/3 the peripheral blood neutrophils of male mice (Peters and Barker). When comparing MRP-14\(^{+/-}\) and MRP-14\(^{-/-}\) mice it was very important to use sex matched mice of the highest backcross.
Chapter 4: Initial characterisation of MRP-14\(^{-/-}\) mice

MRP-14\(^{-/-}\) mice have normal organs and tissues and live a normal lifespan. The architecture of the spleen, where myeloid cells are easily detectable, is unaltered in MRP-14\(^{-/-}\) mice.

4.3.2 Lack of MRP-8 protein in myeloid cells

MRP-14\(^{-/-}\) mice do not express MRP-14 mRNA nor protein. However, although there is normal expression of MRP-8 mRNA in the MRP-14\(^{-/-}\) myeloid cells, surprisingly there is no MRP-8 protein present. Post-transcriptional regulation has been previously reported for S100\(\alpha\) where there is no direct correlation between the levels of S100\(\alpha\) mRNA and protein in rat tissues (Zimmer et al., 1991). Absence of MRP-8 protein could be due to inefficient translation of MPR-8 mRNA, or due to instability of MRP-8 protein in the absence of its partner, MRP-14. To test the latter possibility, I tried adding proteosome inhibitors, (lactacysin or MG132) to bone marrow cells. No MRP-8 was detected in MRP-14\(^{-/-}\) cells. However, this experiment was difficult to interpret because of the lack of a positive control for the effect of the inhibitors. Studies with human MRP-8/14 have demonstrated that MRP-8 in particular is unstable in the absence of its partner (Hunter and Chazin, 1998), supporting the idea that murine MRP-8 protein is unstable and is post-translationally degraded.

The finding that MRP-8 protein is not present in MRP-14\(^{-/-}\) myeloid cells contrasts with evidence that murine MRP-14 and MRP-8 (CP-10) exist separately in myeloid cells (Lackmann et al., 1992; Raftery et al., 1996) and that CP-10 alone can function as a potent chemotactic factor (Lackmann et al., 1992; Lackmann et al., 1993).
Chapter 4: Initial characterisation of MRP-14<sup>−/−</sup> mice

In the MRP-14<sup>−/−</sup> lysate, Ab NH9 recognised one major protein spot corresponding to MRP-8. In addition, 2 minor slightly more acidic spots were detected, one of the same molecular weight and one slightly smaller. These could represent post-translationally modified or truncated isoforms of MRP-8. No alternate forms of murine MRP-8 have been previously reported. Four isoforms of human MRP-8 have been detected in human neutrophil lysates, but they have not been characterised (Guignard et al., 1996).

4.3.3 Lack of compensatory changes in the levels of other proteins in MRP-14<sup>−/−</sup> mice

Murine MRP-14 protein is abundantly expressed, comprising 10-20% of neutrophil soluble protein (Nacken et al., 2000). Similarly, human MRP-8 and 14 proteins constitute 45% of the cytosolic protein of neutrophils and ~1% of monocytes (Edgeworth et al., 1991). It might therefore be expected that the loss of MRP-14 and MRP-8 would be compensated for by an increase in other S100 proteins. S100A4 is expressed in some murine leukocytes (Grigorian et al., 1994) and S100A1 is widely expressed in murine tissues (Du et al., 2002) but expression of both was unaltered in MRP-14<sup>−/−</sup> bone marrow cells (Eileen McNeill, personal communication). Human myeloid cells also express S100A12 (MRP-6, EN-RAGE) (Vogl et al., 1999). However, a search of murine genomic sequences indicates that the mouse does not possess an S100A12-equivalent gene (Robinson, 2000).

Studies of mice deficient in other S100 protein family members have also indicated the lack of compensatory increases in associated S100 family members. S100A1 is abundantly expressed in left ventricular cardiomyocytes at μM
concentrations (Du et al., 2002). In \textit{S100A1}\textsuperscript{−/−} mice, cardiac cells do not upregulate S100 isoforms, S100B, S100A4 or S100A11 which are co-expressed (Du et al., 2002). In \textit{S100B}\textsuperscript{−/−} mice, there is no compensatory upregulation of S100A1 or S100A6 (Nishiyama et al., 2002). Therefore it appears that myeloid and other cell types can develop and function reasonably normally in the absence of their distinctive S100 proteins.

4.3.4 Normal myeloid cell development

Several lines of evidence suggest that myeloid cells from the MRP-14\textsuperscript{−/−} mice are equivalent in maturity to those from the MRP-14\textsuperscript{+/+} mice. Lack of MRP proteins had no effect on the numbers of tissue or circulating leukocytes. There was no difference in the expression of adhesion receptors by MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{−/−} myeloid cells. Moreover, loss of the MRP proteins had no impact on the ultrastructure of the myeloid cells as determined by transmission electron microscopy, which is in keeping with the cytosolic localisation of the proteins (Edgeworth et al., 1991). The MRP proteins therefore have no role in maintaining either the cytoplasmic architecture of myeloid cells or their development or differentiation.

Absence of the MRP proteins resulted in a decrease in mean cellular density of 2.5 mg/ml in MRP-14\textsuperscript{−/−} compared with MRP-14\textsuperscript{+/+} neutrophils. This difference in density had no impact on the flow cytometric scatter properties, confirming that neither the size nor granularity of the cells was different to MRP-14\textsuperscript{−/−} neutrophils. The reduced density of MRP-14\textsuperscript{−/−} neutrophils prevented their purification from bone marrow or blood by Histopaque gradient as they did not localise between the H-1119 and H-1077 phases like MRP-14\textsuperscript{+/+} cells.
4.3.5 Differential regulation of MRP-8 and MRP-14 mRNA expression during embryonic development

MRP-14<sup>−/−</sup> mice have normal organs and tissues and live a normal life span, which is in contrast with MRP-8<sup>−/−</sup> mice that die <i>in utero</i> (Passey et al., 1999a). MRP-8 mRNA is reported to be expressed by fetal cells infiltrating the deciduum, surrounding the ectoplacental cone between 6.5 – 8.5 d.p.c. (Passey et al., 1999a). In confirmation of the findings of Passey <i>et al.</i>, MRP-8 mRNA was found to be expressed at 6.5 and 7.5 d.p.c. in MRP-14<sup>−/+</sup> sections. MRP-14 mRNA was shown for the first time to be expressed in MRP-14<sup>−/+</sup> sections, in a small region of decidual tissue that overlapped with a part of the region of MRP-8 mRNA expression. Therefore, during development, the expression of MRP-8 and MRP-14 mRNA is differentially regulated. MRP-14<sup>−/−</sup> mice express reduced levels of MRP-8 mRNA compared to MRP-14<sup>−/+</sup> mice. This suggests that the expression of MRP-8 mRNA is dependent on the expression of MRP-14. This is in contrast to the expression of MRP-8 in the bone marrow of MRP-14<sup>−/+</sup> mice where MRP-8 is expressed at levels similar to MRP-14<sup>−/+</sup> mice. Therefore the expression of MRP-8 is differentially dependent on MRP-14 expression in different cell types.

4.3.6 Is MRP-8 forming homodimers?

MRP-8 mRNA is expressed in the absence of MRP-14 during development. Although no MRP-8 protein was detected, it is likely that this was due to insensitivity of the immunohistochemistry. If MRP-8 protein is expressed in the absence of MRP-14, it may be forming a homodimer or could be associated with partner other than MRP-14. The ability of murine MRP-8 to form homodimers has
already been demonstrated \textit{in vivo} using the yeast two-hybrid system. Murine MRP-8, but not human MRP-8 and MRP-14 was able to homodimerise, although interaction to form heterodimers was preferred (Propper et al., 1999). It has also been reported that extracellular covalent murine MRP-8 homodimers are formed by oxidation at inflammatory sites (Harrison et al., 1999). However, the oxidised MRP-8 homodimer was deemed non-functional, as it was found to be inactive in chemotaxis assays (Harrison et al., 1999).

\textbf{4.3.7 MRP-8 is expressed by maternal tissue}

Using a fluorescent Y chromosome paint to distinguish maternal and fetal tissue, MRP-8 mRNA expression was found to be associated with maternal rather than fetal tissue. This finding is in disagreement with Passey \textit{et al.}. However, they did not unequivocally distinguish between maternal decidual cells and fetal trophoblasts. The expression of MRP-8 by maternal decidual tissue does not provide an explanation for why MRP-8$^{-/-}$ mice born to MRP-8$^{+/+}$ mothers die in utero ((Passey \textit{et al.}, 1999a), see below).

\textbf{4.3.8 Nature of the maternal cell type expressing MRP-8}

The cell type expressing MRP-8 mRNA in the decidual tissue is currently unknown. As there are very few myeloid cells in the decidual tissue, these cells are not likely to be the major source of MRP-8 mRNA. The timing and localisation of MRP-8 expression in the decidua basalis is very similar to the reported expression of matrix metalloproteinase-3 (MMP-3) (Alexander et al., 1996). MMP-3 expression peaks at 6.5 d.p.c., remains elevated at 7.5 d.p.c. and is undetectable at 8.5 d.p.c. MMP-3 is
expressed in the region of decidual tissue surrounding the embryo. It is expressed in a wave from the antimesometrial pole (below the embryo) to the mesometrial pole (above the e.p.c.). MMP-3 is expressed by differentiating decidual cells. The differentiation of decidual cells is accompanied by a transition in their cellular characteristics from stromal to para-epithelial. The expression of MRP-8 and MRP-14 by this cell type would be consistent with their known expression in epithelial cells in the adult mouse. At the present time, however, the function of MRP-8/14 in both epithelial cells and decidual cells remains a mystery.

4.3.9 MRP-8 is expressed in the labyrinth of the placenta

It has previously been reported that MRP-8 mRNA is expressed by cells associated with the vasculature on the maternal face of the placenta between 10.5-11.5 d.p.c. (Passey et al., 1999a). As Passey et al performed in situ hybridisation on the whole placenta, it was unclear what cell type was expressing MRP-8. The possibility that the placenta had not been stripped of all maternal decidual tissue could not be excluded. In confirmation of these findings, in this chapter MRP-8 mRNA has been shown to be expressed in the placenta at 10.5 but not at 12 or 14.5 d.p.c.. MRP-8 was expressed by the majority of cells within the placental labyrinth. It is likely that these cells are fetal trophoblasts. This should now be confirmed by showing colocalisation of MRP-8 with trophoblast markers and then with markers of specific trophoblast lineages, such as giant cells/spongiotrophoblast/glycogen cells. It will also be interesting to study the expression of MRP-8 by MRP-14<sup>−/−</sup> placental tissue. As MRP-14<sup>−/−</sup> mice are viable, it is likely that MRP-8 will be expressed by MRP-14<sup>−/−</sup> placental tissue.
4.3.10 Why are MRP-8<sup>−/−</sup> mice embryonic lethal?

The finding that MRP-8 is expressed by maternal decidual tissue at 6.5 and 7.5 d.p.c. does not explain the embryonic lethality of MRP-8<sup>−/−</sup> mice at 9.5 d.p.c. born to MRP-8<sup>−/−</sup> mothers (Passey et al., 1999a). Therefore, MRP-8 must also be critically required by cells of fetal origin. The fetal cells expressing MRP-8 may constitute the embryo or the extra-embryonic tissues.

There is a great deal of evidence that supports the idea that MRP-8 expression is required by extra-embryonic fetal cells of the placenta. Firstly, the death of murine embryos around 9.5 d.p.c., as reported for MRP-8<sup>−/−</sup> mice, is a common consequence of defects in extra-embryonic tissues (Cross, 2000). Secondly, as MRP-8 is expressed in the placenta at 10.5 d.p.c., it is possible that MRP-8 is present at earlier time points. It is now important to extend the characterisation of placental expression of MRP-8 to earlier time points, around 8.5 and 9.5 d.p.c. Lastly, there is growing evidence that the trophoblast serves as a component of the innate immune system during pregnancy. For example, in response to *Listeria monocytogenes* infection, trophoblasts secrete the neutrophil chemokines MIP-2 and KC and are essential for controlling the infection (Guleria and Pollard, 2000). The expression of MRP-8 by trophoblasts is consistent with these cells functioning as components of the innate immune system. However, as MRP-8<sup>−/−</sup> embryos die in the absence of infection, MRP-8 must also play an additional role, possibly in placental development (see below).

In order to provide the ultimate proof that MRP-8 expression is absolutely required by extra-embryonic fetal trophoblast cells it will be necessary to rescue the
Chapter 4: Initial characterisation of MRP-14<sup>−/−</sup> mice

Embryonic lethal phenotype of MRP-8<sup>−/−</sup> embryos by tetraploid aggregation (Cross, 2000). In this technique, mutant embryonic stem cells are combined with wild-type tetraploid blastomeres, which contribute to the fetal mesoderm and trophoblast lineage respectively. If the embryo survives, the primary defect lies in the trophoblast lineage. If MRP-8<sup>−/−</sup> mice were viable, this would allow homozygous matings to be set up to determine whether maternal expression of MRP-8 is also essential for development. In addition, the function of MRP-8<sup>−/−</sup> myeloid cells could also be investigated.

4.3.11 What role could MRP-8 be playing during placental development?

MRP-8 is likely to be critical for placental development. It will be important to determine at what stage MRP-8 expression is critical. The study of transgenic mice with placental defects has provided insight into the stages of placental morphogenesis. The murine placenta is derived from two basic elements: an outer epithelium derived from the fetal chorionic plate trophoblast cells, and an underlying vascular network and stroma, which are derived from embryonic allantoic mesoderm. “Chorioallantoic fusion” normally occurs at 8.5 d.p.c.. Several known gene mutations interfere with early chorioallantoic development (Cross, 2000) and cause complete lack of chorionic trophoblasts, produce abnormal allantoic mesoderm outgrowth or prevent chorioallantoic attachment. Other genes are involved in signalling between the allantois and the chorionic trophoblast and are important for formation of the labyrinth of the chorioallantotic placenta from 9.0 d.p.c.. It is unclear at what stage MRP-8 may be playing a critical role.
4.3.12 An additional line of MRP-14" mice

Recent work by Manitz and co-workers reports the characterisation of MRP-14" mice (Manitz et al., 2003). Some basic findings are identical to the MRP-14" mice reported here: MRP-14" mice were viable and showed no obvious phenotype. MRP-8 mRNA was present but MRP-8 protein was absent from MRP-14" peripheral blood cells as determined by flow cytometry. However, Manitz et al. report that MRP-8 protein is present in MRP-14" bone marrow cells at 60% the level of MRP-14" mice, but the composition was restored to 100% during peritonitis, as determined by immunohistochemistry of cytospin preparations. These findings are in complete contrast to those reported in this chapter and I have two criticisms. Firstly, it is unclear why MRP-8 protein expression in bone marrow cells was not studied by flow cytometry, in the same way as peripheral blood leukocytes. Secondly, no negative control is included in the immunohistochemistry figure; positive staining may be due to antibody binding to myeloid cell Fc receptors as the staining protocol did not include an Fc receptor blocking stage.

The differentiation of bone marrow cells in vitro and the numbers of peripheral blood leukocytes cells were normal in MRP-14" mice (Manitz et al., 2003). The number of mAb Gr-1" bone marrow cells over four days of culture was reported to be reduced in MRP-14" mice. However, inspection of the data reveals that there is only a significant difference at day two.

The levels of Mac-1 were studied by flow cytometry. Under resting conditions, MRP-14" Gr-1" bone marrow cells were reported to express reduced levels of Mac-1 in the absence (but not presence) of extracellular Ca²⁺, and were
Chapter 4: Initial characterisation of MRP-14<sup>+/−</sup> mice

unable to upregulate this expression upon IL-8 stimulation. The difference of Mac-1 mean fluorescence of MRP-14<sup>+/−</sup> and MRP-14<sup>+/+</sup> cells was 13 compared to 18 and rose to a maximal level of 22 on MRP-14<sup>+/+</sup> cells. This is an order of magnitude less sensitive than the Mac-1 staining of bone marrow cells reported in this chapter, and may be the result of using mAb M1/70 rather than mAb 5C6. No explanation is given for why the difference in Mac-1 expression is only seen in the absence of extracellular Ca<sup>2+</sup>, and it is unclear what physiological relevance these findings have.
CHAPTER 5

Functional characterisation of MRP-14\(^{-/-}\) myeloid cells.

5.1 Introduction

Murine MRP-14 is abundantly expressed in myeloid cells, comprising 10-20% of neutrophil soluble protein (Nacken et al., 2000). Similarly, human MRP-8 and 14 proteins constitute 45% of the cytosolic protein of neutrophils and 1% of monocytes (Edgeworth et al., 1991). As the MRP proteins have two EF-hand Ca\(^{2+}\) binding motifs, an important issue was whether myeloid cells from MRP-14\(^{-/-}\) mice that lack both MRP-8 and MRP-14, could maintain appropriate cellular levels of Ca\(^{2+}\), as well as generate normal Ca\(^{2+}\) fluxes.

Fluorescent probes have been widely used to study Ca\(^{2+}\) signalling in neutrophils (reviewed in (Hallett et al., 1999; McColl and Naccache, 1997)). There are two main types of Ca\(^{2+}\) probe, those that are monitored at a single wavelength, e.g. Fluo-3, and those that are monitored at two wavelengths (ratiometric dyes), e.g. Fura-2 and Indo-1. As single wavelength indicators only produce a single intensity change on binding Ca\(^{2+}\), caution must be exercised when interpreting changes in fluorescence intensity. For example, a decrease in fluorescence could result from a decrease in the concentration of probe, presence of a quenching agent or a decreased
Ca$^{2+}$ concentration. However, by using a ratiometric dye, there can be certainty that the ratio change is due to a Ca$^{2+}$ signal.

Another important dye parameter is the dissociation constant, $K_d$. The greatest change in Ca$^{2+}$ binding to the probe occurs at changes in Ca$^{2+}$ around the $K_d$ value. The $K_d$ for Indo-1 and Fura-2 are 250 nM and 224 nM respectively, which are much less than Fluo-3 whose $K_d$ is 864 nM. As levels of cytosolic free Ca$^{2+}$ in resting neutrophils are around 100 nM, Indo-1 and Fura-2 are preferable to Fluo-3. Indo-1 has several advantages over Fura-2. Firstly, Indo-1 has a lower affinity for Ca$^{2+}$ than Fura-2, therefore fast Ca$^{2+}$ transients can be more readily visualised. Secondly, the fluorescence of Indo-1 is measured at 405/485 nm, rather than 340/380 nm for Fura-2, which reduces the noise due to neutrophil autofluorescence. Lastly, Indo-1 fluorescence can be monitored by flow cytometry, this allows measurement of Ca$^{2+}$ within subpopulations of cells.

Changes in intracellular Ca$^{2+}$ play a fundamental role in many key neutrophil activities (reviewed in (Pettit et al., 1997), including in degranulation, phagocytosis, superoxide burst, apoptosis and signalling by many seven-transmembrane domain receptors. MRP-8/14 has also been directly implicated in the superoxide burst. MRP-8/14 have been shown to enhance the activation of the neutrophil NADPH oxidase (Doussiere et al., 2001; Doussiere et al., 2002), although the mechanism is not understood. In addition, MRP-8/14 is translocated upon cellular activation to the plasma membrane (Lemarchand et al., 1992), the site of superoxide production, phagocytosis and large changes in Ca$^{2+}$ concentration (Davies and Hallett, 1998), although its role remains unclear.
Chapter 5: Functional characterisation of MRP-14<sup>+</sup> myeloid cells

The aim of this part of the project was to examine the Ca<sup>2+</sup> responses and functions of MRP-14<sup>+</sup> myeloid cells.
5.2 Results

5.2.1 Source of murine myeloid cells

The finding that endotoxin contamination was responsible for the effects of rMRP-14 in vivo, led me to eliminate other potential sources of endotoxin. Low endotoxin media and chemicals were used for all assays. However, when very low endotoxin FCS (Alexander Edwards, personal communication) was used in the culture medium to prepare "mature" granulocytes, the cells no longer became fMLP responsive (data not shown). This suggested that the "maturation" of granulocytes was likely to be due to endotoxin contamination of FCS. As these activated cells may not best resemble the function of myeloid cells in vivo, fresh bone marrow cells were used for in vitro assays.

Bone marrow neutrophils had been previously purified from bone marrow using a Histopaque gradient, but as MRP-14^- neutrophils were less dense than MRP-14^+ cells (Eileen McNeill, personal communication), neutrophils could no longer be separated using this method. Therefore, a number of flow cytometric assays were developed to test MRP-14^- myeloid cell function in a mixed bone marrow cell population.

5.2.2 Elevated basal intracellular Ca^{2+} levels in MRP-14^- neutrophils

The basal level of intracellular Ca^{2+} in MRP-14^+/+ and MRP-14^-/- neutrophils was measured by flow cytometry. MRP-14^+/+ and MRP-14^-/- bone marrow cells were
Chapter 5: Functional characterisation of MRP-14<sup>+</sup> myeloid cells

loaded with Indo-1 and neutrophils were identified by staining with mAbs 7/4 and Gr-1 (Henderson et al., 2003). The absolute level of intracellular Ca<sup>2+</sup> was quantified using the procedure outlined in Figure 5.1. Extracellular Ca<sup>2+</sup> was chelated immediately prior to recording, then minimum and maximum fluorescences where achieved by addition of ionomycin followed by excess Ca<sup>2+</sup>. The basal level of intracellular Ca<sup>2+</sup> in MRP-14<sup>-</sup> and MRP-14<sup>+</sup> neutrophils was 31.0 and 22.3 nM respectively. Over the course of a 2 hour Ca<sup>2+</sup> flux experiment, basal levels of intracellular Ca<sup>2+</sup> increased slowly by around 20 – 30 nm, but the difference between MRP-14<sup>+</sup> and MRP-14<sup>-</sup> neutrophils was consistently maintained.

5.2.3 Role of MRP-14 in chemoattractant induced Ca<sup>2+</sup> responses

The ability of MRP-14<sup>+/+</sup> and MRP-14<sup>-/-</sup> neutrophils to flux Ca<sup>2+</sup> in response to the bacterial formylated peptide fMLP, in the presence and absence of extracellular Ca<sup>2+</sup> was tested (Figure 5.2). MRP-14<sup>+/+</sup> and MRP-14<sup>-/-</sup> neutrophil responses were identical. fMLP caused a Ca<sup>2+</sup> flux from a threshold level of 0.1 μM to a peak response at 10 μM. In the presence of extracellular Ca<sup>2+</sup>, the initial increase in intracellular Ca<sup>2+</sup> was identical to the response in the absence of extracellular Ca<sup>2+</sup>, but after 2 minutes the Ca<sup>2+</sup> response was more sustained.
Figure 5.1 Calibration of intracellular Ca\(^{2+}\) levels
Bone marrow leukocytes were loaded with Indo-1 and neutrophils were identified by labelling with mAbs 7/4 and Gr-1. Intracellular Ca\(^{2+}\) was monitored by flow cytometry in the presence of EGTA to chelate extracellular Ca\(^{2+}\). The fluorescence at 424 nm (FL-4) corresponds to Ca\(^{2+}\)-free Indo-1 and the fluorescence at 530 nm (FL-5) corresponds to Ca\(^{2+}\)-bound Indo-1. Autofluorescence has been subtracted from all values of FL-4/5 fluorescence. The indo-1 ratio (R) is the ratio of FL-5/FL-4 fluorescence. The levels of intracellular Ca\(^{2+}\) are calculated using the equation shown, using \(K_d\), the dissociation constant for Indo-1 (\(K_d = 250\) nM), and the minimum (\(R_{\text{min}}\)) and maximum (\(R_{\text{max}}\)) Indo-1 ratios obtained by adding ionomycin (10 \(\mu\)M) in the presence of EGTA and Ca\(^{2+}\) (10 mM) respectively.

\[
\begin{align*}
\text{Indo-1 ratio (R) &= FL-5 / FL-4} \\
R_{\text{min}} &= FL-5_{\text{A}} / FL-4_{\text{A}} \\
R_{\text{max}} &= FL-5_{\text{B}} / FL-4_{\text{B}} \\
S &= FL-4_{\text{A}} / FL-4_{\text{B}} \\
[\text{Ca}^{2+}] &= K_d \times \frac{(R - R_{\text{min}}) \times S}{(R_{\text{max}} - R)}
\end{align*}
\]
Figure 5.2 Normal fMLP Ca$^{2+}$ response of MRP-14$^{+}$ neutrophils
MRP-14$^{+/+}$ (green) and MRP-14$^{-/-}$ (red) bone marrow leukocytes were loaded
with Indo-1 and neutrophils were identified by labelling with mAbs 7/4 and
Gr-1. Intracellular Ca$^{2+}$ was monitored by flow cytometry and was expressed
as the ratio of the Indo-1 fluorescence at 424 nm and 530 nm. The median
neutrophil Indo-1 ratio of $\approx$ 500-700 events is plotted each second. (A) The
response of neutrophils to the chemoattractant fMLP in the presence of
extracellular Ca$^{2+}$. (B) The response of neutrophils to the chemoattractant
fMLP in the presence of EGTA to chelate extracellular Ca$^{2+}$. Data representative of 3 experiments.
Chapter 5: Functional characterisation of MRP-14<sup>+/−</sup> myeloid cells

The ability of MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils to flux Ca<sup>2+</sup> in response to the chemokine, MIP-2 was tested in the presence or absence of extracellular Ca<sup>2+</sup> (Figure 5.3 A,B). MIP-2 produced a Ca<sup>2+</sup> flux in MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils from a threshold level of 0.5 ng/ml to a peak response at 10 ng/ml. At maximal doses of MIP-2, the levels of intracellular calcium in MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils were identical, but at lower concentrations of stimulant, the response of MRP-14<sup>−/−</sup> neutrophils was significantly diminished. For example, the change in intracellular calcium levels produced by 1 ng/ml MIP-2 in MRP-14<sup>−/−</sup> neutrophils was 40% lower than MRP-14<sup>+/−</sup> cells (Fig. 5.3 C). The rate of decay of the Ca<sup>2+</sup> transients was similar for MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils in the presence of extracellular Ca<sup>2+</sup>. The small difference in the rate of decay between MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils in the absence of extracellular Ca<sup>2+</sup> was not reproducible.

5.2.4 Normal Ca<sup>2+</sup> release from stores and across the plasma membrane in MRP-14<sup>−/−</sup> neutrophils

To examine the release of Ca<sup>2+</sup> from intracellular stores, the inhibitor thapsigargin was used. Thapsigargin inhibits the ATP-dependent Ca<sup>2+</sup> pump on intracellular stores, causing the rapid leakage of Ca<sup>2+</sup> into the cytoplasm. In the presence of extracellular Ca<sup>2+</sup>, Ca<sup>2+</sup> release from stores can stimulate Ca<sup>2+</sup> influx across the plasma membrane, through a poorly understood mechanism. Thapsigargin induced a similar increase in intracellular Ca<sup>2+</sup> in MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils in the presence or absence of extracellular Ca<sup>2+</sup> (Figure 5.4 A,B).
Figure 5.3 Reduced MIP-2 \( \text{Ca}^{2+} \) response in MRP-14\(^{+/+}\) neutrophils

MRP-14\(^{+/+}\) (green) and MRP-14\(^{-/-}\) (red) bone marrow leukocytes were loaded with Indo-1 and neutrophils were identified by labelling with mAbs 7/4 and Gr-1. Intracellular \( \text{Ca}^{2+} \) was monitored by flow cytometry and was expressed as the ratio of the Indo-1 fluorescence at 424 nm and 530 nm. The median neutrophil Indo-1 ratio of \( \approx 500-700 \) events is plotted each second. (A) The response of neutrophils to the chemokine MIP-2 in the presence of 1 mM extracellular \( \text{Ca}^{2+} \). (B) The response of neutrophils to the chemokine MIP-2 in the presence of EGTA to chelate extracellular \( \text{Ca}^{2+} \). (C) Maximal changes in intracellular \( \text{Ca}^{2+} \) levels induced by suboptimal concentrations of MIP-2 in the presence of extracellular \( \text{Ca}^{2+} \) are significantly lower in MRP-14\(^{-/-}\) cells. Data are reported as mean ± SEM, \( p < 0.05 \). Data representative of 6 experiments.
Figure 5.4 Normal Ca^{2+} release from stores and influx across the plasma membrane in MRP-14^{-/-} neutrophils.
MRP-14^{+/+} (green) and MRP-14^{-/-} (red) bone marrow leukocytes were loaded with Indo-1 and neutrophils were identified by labelling with mAbs 7/4 and Gr-1. Intracellular Ca^{2+} was monitored by flow cytometry and was expressed as the ratio of the Indo-1 fluorescence at 424 nm and 530 nm. The median neutrophil Indo-1 ratio of ∼ 500-700 events is plotted each second. (A) The response of neutrophils to 5 μM thapsigargin in the presence of 1 mM extracellular Ca^{2+}. (B) The response of neutrophils to 5 μM thapsigargin in the presence of EGTA, to chelate extracellular Ca^{2+}. (C) The response of neutrophils to 1 μM ionomycin in the presence of 1 mM extracellular Ca^{2+}. Data representative of 3 experiments.
Chapter 5: Functional characterisation of MRP-14<sup>+/−</sup> myeloid cells

To test the influx of Ca<sup>2+</sup> across the plasma membrane, the Ca<sup>2+</sup> ionophore, ionomycin, was used. In the presence of extracellular Ca<sup>2+</sup>, ionomycin caused a rapid influx of Ca<sup>2+</sup> into the cytosol of MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils with similar magnitude and kinetics (Figure 5.4 C).

5.2.5 Normal chemotactic response of MRP-14<sup>−/−</sup> neutrophils

The ability of neutrophils to migrate from the vasculature into tissue was modelled in vitro using the Transwell chemotaxis assay. Bone marrow neutrophils were allowed to migrate towards MIP-2 over a concentration range of 0.1-500 ng/ml. MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils showed a similar bell shaped curve of responsiveness (Figure 5.5). There was also no difference in the chemotactic response to MIP-2 when cells migrated across a TNF-α-stimulated bEND5 endothelial monolayer (Kiki Tanousis, personal communication).

5.2.6 Normal superoxide burst response of MRP-14<sup>−/−</sup> neutrophils

MRP-8/14 has been shown to enhance the activation of the superoxide burst by phorbol ester (Doussiere et al., 2001). Therefore the ability of MRP-14<sup>−/−</sup> neutrophils to perform this activity was tested. Induction of a respiratory burst was measured by flow cytometry using dihydrorhodamine (DHR), a probe that is taken up by cells and becomes fluorescent upon oxidation. There was no difference in production of oxygen radicals by MRP-14<sup>+/−</sup> and MRP-14<sup>−/−</sup> neutrophils in response to a range of concentrations of the phorbol esters PMA and PDBu (Figure 5.6 ).
Figure 5.5 Normal chemotactic response of MRP-14<sup>−/−</sup> neutrophils towards MIP-2.
Migration of MRP-14<sup>−/−</sup> (green bars) and MRP-14<sup>−/+</sup> (red bars) bone marrow neutrophils in a Transwell chemotaxis assay in response to the chemokine MIP-2. Cells were allowed to migrate for 2 hours, then cells in the bottom well were stained with mAbs 7/4 and Gr-1. The number of migrated neutrophils was determined by flow cytometry. Data are expressed as mean ± SD. Data representative of 4 experiments.
Figure 5.6 Normal superoxide burst response of MRP-14+/ neutrophils
MRP-14+/ (green line) and MRP-14- (red line) bone marrow leukocytes
were incubated with DHR for 5 minutes prior to the addition of phorbol
esters, PMA or PDBu, or medium alone. After 30 minutes stimulation,
neutrophils were identified by labelling with mAbs 7/4 and Gr-1 and the
production of intracellular oxygen radicals was detected by the oxidation of
DHR to a fluorescent derivative. Inset numbers: DHR geometric mean
fluorescence. Data representative of 3 experiments.
5.2.7 Normal apoptosis response of MRP-14^- myeloid cells

Neutrophil apoptosis serves to limit tissue damage caused by these cells and plays an important role in the resolution of the inflammatory response. The ability of MRP-14^- neutrophils to undergo apoptosis in response to a variety of stimuli was tested. Apoptosis was detected by staining cells with LDS-751, a nucleic acid binding dye. LDS-751 reports the state of chromatin and binds less to condensed chromatin. Therefore apoptotic cells stain less than normal cells. LDS-751 was used in combination with DAPI, a nucleic acid binding dye that is excluded from living cells, to discriminate live apoptotic and dead cells. This technique is illustrated in Figure 5.7.

To examine the spontaneous death of MRP-14^- myeloid cells, bone marrow cells were cultured for up to 4 days in the presence or absence of FCS and the number of live/apoptotic/dead cells were measured. The death of MRP-14^- and MRP-14^- neutrophils in culture followed very similar kinetics. After one day of culture in the absence of FCS, the numbers of live neutrophils fell to less than half the starting numbers, and the majority had disappeared after two days (Figure 5.8 A). In the presence of FCS, cells lived slightly longer, approximately a quarter of the cells were still alive after two days in culture, but the vast majority were dead a day later (Figure 5.8 A).
Figure 5.7 Measurement of neutrophil and monocyte apoptosis

(A) Bone marrow cells were labelled with mAbs 7/4 and Gr-1 to identify neutrophils (7/4+ Gr-1+; purple gate, R1) and monocytes (7/4+ Gr-1-; R2).

(B) In addition, cells were labelled with LDS-751 and DAPI to discriminate live (LDS+ DAPI+), apoptotic (LDS**(dim)** DAPI+) and dead (DAPI+) cells.

(C) Typical results showing the response of bone marrow neutrophils (R1) to 4 hr culture in medium alone, thapsigargin (2.5 μM) or staurosporine (STS) (1 μM) to demonstrate populations that mainly consist of live, apoptotic and dead cells, respectively.
Figure 5.8 Spontaneous and TNF-α / gliotoxin-induced apoptosis and death of bone marrow neutrophils

(A) MRP-14+/+ and MRP-14−/− bone marrow cells were incubated for 0 - 4 days in DMEM ± FCS. Cells were then labelled with mAbs 7/4 and Gr-1, and LDS-751 and DAPI to identify live/apoptotic/dead neutrophils. Data representative of 3 experiments.

(B) MRP-14+/+ and MRP-14−/− bone marrow cells were incubated for 6 hours with TNF-α (10 ng/ml) ± gliotoxin (Glio) at a range of concentrations, staurosporine (S; 1 μM), or medium alone. Cells were then labelled with mAbs 7/4 and Gr-1, and LDS-751 and DAPI to identify live/apoptotic/dead neutrophils. Data representative of 2 experiments.
Chapter 5: Functional characterisation of MRP-14<sup>−/−</sup> myeloid cells

TNF-α has been reported to exert differential effects on neutrophil apoptosis, accelerating apoptosis at early time points and inhibiting it at later time points (Ward et al., 1999b). The fungal metabolite, gliotoxin, has been shown to increase the rate of constitutive apoptosis and dramatically increase the pro-apoptotic effects of TNF-α in human neutrophils (Ward et al., 1999a). The ability of TNF-α and gliotoxin to induce apoptosis in MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> bone marrow neutrophils was tested. The response of MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> neutrophils was similar (Figure 5.8 B). TNF-α caused a small increase in the percentage of apoptotic cells, and gliotoxin caused an increase in the percentage of dead cells with a bell shaped curve of responsiveness. The effects of TNF-α and gliotoxin were additive, but not synergistic as seen with human neutrophils (Ward et al., 1999a).

Agents that directly mobilise Ca<sup>2+</sup> can trigger apoptosis in a variety of cell types (McConkey and Orrenius, 1997) and inhibitors of protein kinase C, e.g. staurosporine, have been demonstrated to promote neutrophil apoptosis (Cousin et al., 1997). The ability of these stimuli to induce apoptosis in MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> bone marrow myeloid cells was tested over an 8 hour time course. There was no difference in the proportions of live/apoptotic/dead neutrophils (Figure 5.9 A) or monocytes (Figure 5.9 B) of MRP-14<sup>+/+</sup> and MRP-14<sup>−/−</sup> mice for any of the stimuli. Ionomycin increased the percentage of apoptotic neutrophils and monocytes at both 4 hours and 8 hours compared to cells that were incubated in medium alone. Thapsigargin and staurosporine both increased the percentages of apoptotic and dead cells by 4 hours and by 8 hours the majority of the cells were dead.
Figure 5.9 Apoptosis and death of bone marrow myeloid cells in response to staurosporine and Ca^{2+} mobilisers

MRP-14^{+/+} and MRP-14^{-/-} bone marrow cells were incubated for 4 or 8 hours with staurosporine (S; μM), ionomycin (Iono; 1 μM), thapsigargin (Tg; 2.5 μM) or medium alone. Cells were then labelled with mAbs 7/4 and Gr-1, and LDS-751 and DAPI to identify live/apoptotic/dead neutrophils and monocytes. Data representative of 4 experiments.
5.3 Discussion

5.3.1 Elevated basal intracellular $\text{Ca}^{2+}$ levels in MRP-14$^{+/}$ neutrophils

Intracellular $\text{Ca}^{2+}$ levels in MRP-14$^{+/}$ neutrophils were measured by flow cytometry. The advantages of this technique are that the $\text{Ca}^{2+}$ flux response of 1000s of cells can be simultaneously measured and the median values calculated. In addition, $\text{Ca}^{2+}$ responses in distinct subpopulations of cells can be measured. Therefore this technique is particularly suitable for studying bone marrow neutrophils, which are in a mixed cell population and may be at different stages of development. Using this technique, the basal level of intracellular $\text{Ca}^{2+}$ in MRP-14$^{+/}$ neutrophils was found to be consistently higher than that of MRP-14$^{+/-}$ cells. Elevations in the basal levels of intracellular $\text{Ca}^{2+}$ in MRP-14$^{+/}$ neutrophils could either be due to the failure of a system removing cytosolic $\text{Ca}^{2+}$ or activation of a $\text{Ca}^{2+}$ entry pathway. In resting cells, $\text{Ca}^{2+}$ is continually removed from the cytosol by $\text{Ca}^{2+}$ ATPases in the plasma membrane and the ER. The plasma membrane ATPase plays the most important role in maintaining the gradient of $\text{Ca}^{2+}$ across the plasma membrane, whereas the ER ATPase is responsible for the fine regulation of cytosolic $\text{Ca}^{2+}$. As the levels of intracellular $\text{Ca}^{2+}$ are only slightly elevated in MRP-14$^{+/}$ neutrophils, it is possible that this may be due to problems with the ER ATPase.

An alternative explanation for the elevated baseline levels of intracellular $\text{Ca}^{2+}$ in MRP-14$^{+/}$ neutrophils may be explained by MRP-14's role as the major arachidonic acid binding protein in neutrophils (Kerkhoff et al., 1999a). Arachidonic
acid has been shown to affect the intracellular calcium concentration in many cell types (Striggow and Ehrlich, 1997) and recently an arachidonic acid regulated non-capacitative Ca\(^{2+}\) entry pathway has been identified in non-excitable cells (Mignen and Shuttleworth, 2000). Therefore, alterations in arachidonic acid metabolism or transport in MRP-14\(^{-/-}\) cells may cause the activation of such Ca\(^{2+}\) entry pathways and the elevated basal Ca\(^{2+}\) levels.

5.3.2 Reduced responsiveness of MRP-14\(^{-/-}\) neutrophils to MIP-2 but not fMLP

There was no difference in the Ca\(^{2+}\) flux response of MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) neutrophils to the bacterial chemoattractant fMLP, over a range of concentrations from 0.1 – 10 μM. The Ca\(^{2+}\) flux responses to maximal levels of the neutrophil chemokine MIP-2, were also similar between MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) neutrophils. However, at suboptimal levels of MIP-2, the Ca\(^{2+}\) response of MRP-14\(^{-/-}\) neutrophils was significantly diminished by approximately 40% compared with MRP-14\(^{+/+}\) cells. This difference was not due to problems with Ca\(^{2+}\) storage within organelles, as a similar amount of intracellular Ca\(^{2+}\) was released from stores by thapsigargin in MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) neutrophils. Every care was taken to ensure that this difference in MIP-2 sensitivity was not due to strain differences between C57BL/6 and 129 mice. For Ca\(^{2+}\) flux experiments sex matched littermates were always compared, and this difference in Ca\(^{2+}\) flux was repeated many times with mice at each successive backcross level as they became available.

MRP-8/14 is known to translocate to the plasma membrane in a Ca\(^{2+}\) dependent manner (Lemarchand et al., 1992). At the plasma membrane MRP-14 may activate a component of the MIP-2 signalling cascade to stimulate the Ca\(^{2+}\) response.
Chapter 5: Functional characterisation of MRP-14\(^+\) myeloid cells

MRP-14 may act directly on the MIP-2 receptor, this would explain the specificity of the defect in MIP-2 signalling in MRP-14\(^+\) neutrophils. Alternatively, MRP-14 may be involved in regulating the pathway downstream of the MIP-2 receptor. fMLP and MIP-2 signalling pathways have many features in common. Both fMLP and MIP-2 bind to seven transmembrane domain receptors, which are coupled to G\(_i\) proteins, a class of G protein that is sensitive to Pertussis toxin. Downstream of G\(_i\) proteins, two pathways have been characterised, which involve the metabolism of phosphatidylinositides, particularly phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)) (reviewed in (Wu et al., 2000)). Phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) are activated by the G\(_{\beta\gamma}\) subunits released from the G\(_i\) proteins, and hydrolyse and phosphorylate PIP\(_2\), respectively. Neutrophils contain two PLC isoforms, PLC\(_{\beta2}\) and PLC\(_{\beta3}\). The importance of these isoforms in neutrophil chemoattractant Ca\(^{2+}\) responses has been demonstrated using PLC\(_{\beta2}\)\(^{-/-}\) PLC\(_{\beta3}\)\(^{-/-}\) double knock out mice (Li et al., 2000). These mice are unable to respond to IL-8 or fMLP in Ca\(^{2+}\) flux assays. Therefore, the signalling pathways downstream of fMLP and MIP-2 receptors both involve the activation of PLC\(_{\beta2}\) by G\(_i\) proteins, however there are significant differences between these two pathways.

5.3.3 Differences between fMLP and MIP-2 signalling pathways

Cyclic ADP-ribose gated Ca\(^{2+}\) channels appear to be an essential and specific transducer of fMLP, but not MIP-2 signals in mouse neutrophils (Partida-Sanchez et al., 2001). CD38, is a transmembrane glycoprotein that catalyses the production of cyclic ADP-ribose from its substrate, NAD\(^+\). Cyclic ADP-ribose regulates intracellular Ca\(^{2+}\) release from ryanodine receptor regulated stores that are distinct
from those controlled by IP$_3$. Two lines of evidence suggest that this pathway is important in the fMLP but not IL-8 (the human homologue of MIP-2) response of neutrophils (Partida-Sanchez et al., 2001). Firstly, the magnitude of the Ca$^{2+}$ flux stimulated by fMLP was 20% lower in CD38$^{-/-}$ compared to CD38$^{+/+}$ neutrophils and the influx of extracellular Ca$^{2+}$ was essentially abolished in CD38$^{-/-}$ neutrophils. The IL-8 response was unaffected in CD38$^{+/+}$ cells. Secondly, the Ca$^{2+}$ response CD38$^{+/+}$ neutrophils to fMLP, could be reduced to CD38$^{-/-}$ levels by pretreatment with a cyclic ADP-ribose antagonist, 8-Br-cADPR. However, the response to IL-8 was not affected by 8-Br-cADPR, nor inhibitors of the ryanodine receptors, ryanodine and ruthenium red (Schorr et al., 1999). In summary, this evidence suggests that cyclic ADP-ribose regulates Ca$^{2+}$ release and extracellular Ca$^{2+}$ influx in response to fMLP but not IL-8. This is consistent with the fact that only fMLP causes subsequent Capacitative Ca$^{2+}$ entry (Partida-Sanchez et al., 2001).

As fMLP opens both IP$_3$ and cyclic ADP-ribose gated stores but MIP-2 only opens IP$_3$ gated stores, the hyporesponsiveness of MRP-14$^{-/-}$ neutrophils to low levels of MIP-2 could be due to a partial defect in the IP$_3$ pathway. This effect may be masked and not cause a problem during fMLP signalling as this pathway uses an additional mechanism of Ca$^{2+}$ release. For example, MRP-14 may be important for the optimal activation of PLC$\beta$. This is consistent with the translocation of MRP-14 to the plasma membrane on cellular activation. A positive feedback loop of PLC activation could be imagined in which IP$_3$ produced by PLC causes the release of Ca$^{2+}$ that binds to MRP-14 causing it to translocate to activate PLC$\beta$ and cause further IP$_3$ production. In MRP-14$^{-/-}$ neutrophils, this feedback loop would be absent and could explain the reduced Ca$^{2+}$ release from stores in these cells. Another
possibility is that MRP-14 is somehow involved with the release of Ca\(^{2+}\) from stores. The distribution of MRP-14 throughout the cytoplasm or at the plasma membrane is not consistent with a direct role in regulating IP\(_3\) gated Ca\(^{2+}\) channels. It is known that the kinetics of IP\(_3\) gated Ca\(^{2+}\) release are profoundly affected by Ca\(^{2+}\) ions (Dawson, 1997) and the elevated basal level of intracellular Ca\(^{2+}\) in MRP-14\(^{-/-}\) neutrophils may somehow have an inhibitory effect on Ca\(^{2+}\) release. Alternatively, the IP\(_3\) receptor may be partially inhibited in MRP-14\(^{-/-}\) neutrophils indirectly, through MRP-14’s role as the major arachidonic acid binding protein in neutrophils (Kerkhoff et al., 1999a). Phospholipase A\(_2\) is activated in response to many agonists and catalyses the release of arachidonic acid, which has been shown to inhibit the IP\(_3\) receptor but not the ryanodine receptor (Striggow and Ehrlich, 1997). Therefore, alterations in arachidonic acid metabolism or transport in MRP-14\(^{-/-}\) cells may explain the inhibition of IP\(_3\) gated Ca\(^{2+}\) release.

There is increasing evidence that chemokine receptors may activate receptor-specific and G-protein independent signal transduction pathways, although the molecular basis has yet to be determined (see (Thelen, 2001) and references therein). For example CXCR1 stimulates phospholipase D activation and superoxide production whereas CXCR2 does not trigger either response. In addition chemokine receptor phosphorylation and internalisation is not inhibited by pertussis toxin. Therefore MRP-14 may be important for regulating an as yet undiscovered specific component of the MIP-2 signalling cascade.
Chapter 5: Functional characterisation of MRP-14−/− myeloid cells

5.3.4 MRP-14 as a Ca^{2+} sensor

The observation that MRP-14−/− neutrophils are less responsive to MIP-2 is consistent with the interpretation that MRP-8/14 acts as a Ca^{2+} sensor, and not a Ca^{2+} buffer, as loss of a Ca^{2+} buffer would be expected to enhance Ca^{2+} transients. Further evidence that MRP-8/14 is acting as a calcium sensor comes from physical studies of MRP-8/14 demonstrating conformational changes following Ca^{2+} binding by the complex (Hunter and Chazin, 1998). In addition, analysis of crystal structures of S100A6 (Otterbein et al., 2002) and S100B (Drohat et al., 1998) in the presence or absence of Ca^{2+} shows conformational change in the S100 dimer. This conformational change leads to the exposure of a hydrophobic patch on each monomer that provides a docking site for interaction with other proteins, thereby propagating the Ca^{2+} signal. There are now a number of situations where such Ca^{2+}-induced changes in S100 proteins have been demonstrated to have biological relevance. For example, S100B inhibits p53 phosphorylation by protein kinase C in a Ca^{2+}-dependent fashion (Baudier et al., 1992). The giant kinase twitchin is activated by S100A1 in a Ca^{2+}/Zn^{2+} dependent manner (Heierhorst et al., 1996). S100B activates photoreceptor guanylate cyclase (Duda et al., 2002) and also the nuclear Ndr serine/threonine kinase with dependence on Ca^{2+} (Millward et al., 1998).

5.3.5 Normal chemotactic response and superoxide burst of MRP-14−/− neutrophils

The diminished level of Ca^{2+} response to MIP-2 in MRP-14−/− neutrophils had no impact on their chemotactic response to MIP-2. The signalling pathways that link chemoattractant receptors to chemotaxis are not well understood and it is still
Chapter 5: Functional characterisation of MRP-14\textsuperscript{−/−} myeloid cells

controversial as to whether Ca\textsuperscript{2+} is absolutely required for chemotaxis (Hallett, 1997). Neutrophils isolated from PLC\textsubscript{β2}\textsuperscript{−/−} PLC\textsubscript{β3}\textsuperscript{−/−} double knock out mice do not show any defects in chemokine-stimulated migration, which suggests that Ca\textsuperscript{2+} elevations may not be necessary for directional sensing and shape change (Li et al., 2000). In addition, Ca\textsuperscript{2+} signals triggered by a sudden change in the concentration of a stimulus in a Ca\textsuperscript{2+} flux assay do not mimic the gradual change in concentration that drives chemotaxis.

The production of oxygen free radicals by the NADPH oxidase is essential for bacterial killing by neutrophils. Phorbol esters directly activate PKC and induce the assembly of an active NADPH oxidase complex at the plasma membrane and in granules (Lundqvist et al., 1995). MRP-8/14 has been reported to enhance the activation of the superoxide burst by phorbol ester (Doussiere et al., 2001) and has been shown to interact directly with oxidase components \textit{in vitro} (Doussiere et al., 2002). There was no difference in the intracellular production of oxygen radicals by MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{−/−} neutrophils stimulated by phorbol esters. Preliminary results measuring the kinetics of phorbol ester-stimulated oxygen radical production at the plasma membrane using cytochrome C reduction also did not reveal any difference in the MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{−/−} neutrophils (data not shown). Therefore MRP-14 appears to be dispensible for activation of the superoxide burst by phorbol ester \textit{in vivo}. The neutrophil respiratory burst can be activated by a number of physiological stimuli, including chemoattractants and certain cytokines (Dahlgren and Karlsson, 1999), which stimulate signalling pathways that activate the oxidase. As the ability of MRP-14\textsuperscript{−/−} neutrophils to superoxide burst in response to these stimuli was not tested, it cannot be excluded that MRP-14 may be important in the
pathway linking G-protein coupled receptors to the activation of the NADPH oxidase.

The ability of MRP-14" neutrophils to phagocytose E.coli has been shown to be similar to MRP-14"/+ cells (Meg Mathies, personal communication). It will be important to test the ability of MRP-14" neutrophils to kill live bacteria.

5.3.6 Normal spontaneous and receptor mediated apoptosis of MRP-14" myeloid cells

LDS-751 was used to measure apoptosis in MRP-14"/+ and MRP-14"- myeloid cells. LDS-751 was preferable to annexin V to measure apoptosis as the changes in chromatin detected by LDS-751 occur rapidly, at an earlier stage in apoptosis than the breakdown in plasma membrane asymmetry reported by annexin V (Frey, 1997). The kinetics of cell death in culture was similar for MRP-14"/+ and MRP-14"- neutrophils. Surprisingly, the decrease in the number of live cells was not accompanied by an increase in the number of apoptotic or dead cells. This is probably due phagocytosis of apoptotic/dying neutrophils by other cells in the mixed bone marrow population.

Apoptosis was also induced in MRP-14"/+ and MRP-14"- myeloid cells using a variety of stimuli that signal via different pathways. Staurosporine, gliotoxin and TNF-α induce apoptosis via PKC, NF-κB and the caspase/NF-κB/p38-Jun N-terminal kinase pathways respectively (Gordge and Ryves, 1994; Ward et al., 1999a; Ward et al., 1999b). There was no difference in the response of MRP-14"/+ and MRP-14"- myeloid cells to these stimuli.
Gliotoxin has been reported to act synergistically with TNF-α to promote apoptosis of human neutrophils (Ward et al., 1999a). TNF-α and gliotoxin were not synergistic in promoting the apoptosis of murine bone marrow neutrophils, instead their effects were only additive. There were many other differences in the response of human and myeloid cells to gliotoxin. Human neutrophils were approximately 10 times more sensitive to gliotoxin, and apoptosis was induced much more rapidly in these cells (2 hour vs 6 hours). Gliotoxin mainly caused murine neutrophil cell death. However, the ability of gliotoxin to cause death of human neutrophils was not tested, for example by using a live/dead indicator such as DAPI. In summary, there were large differences in the response of human and murine myeloid cells to gliotoxin. This may be due to species differences or differences between peripheral blood and bone marrow myeloid cells.

5.3.7 Normal apoptosis of MRP-14/+ myeloid cells in response to Ca²⁺ mobilisers

The Ca²⁺ mobilisers ionomycin and thapsigargin induced apoptosis and death to a similar extent in MRP-14/+ and MRP-14−/− neutrophils. Ionomycin-induced neutrophil apoptosis has been reported to be mediated through the intracellular production of oxygen radicals (Lundqvist-Gustafsson and Bengtsson, 1999). This idea is supported by the finding that ionomycin did not cause apoptosis in dimethyl sulphoxide-differentiated HL-60 cells, which are devoid of specific granules containing NADPH oxidase (Lundqvist-Gustafsson and Bengtsson, 1999). The cytotoxicity of agents that generate reactive oxygen species is thought to be mediated by damage to Ca²⁺ transport systems within the ER, mitochondria and plasma
Chapter 5: Functional characterisation of MRP-14<sup>−/−</sup> myeloid cells

membrane, which leads to apoptosis (Ermak and Davies, 2001). Thapsigargin, like ionomycin, caused apoptosis of bone marrow neutrophils. This finding conflicts with a previously study that reported that thapsigargin inhibits human peripheral blood neutrophil apoptosis (Cousin et al., 1997). However, there were a number of important differences between these assays. Firstly thapsigargin was incubated with human cells for 20 hours, rather than up to 8 hours for the murine cells. In addition, the response to thapsigargin may vary between different cell types and species. The finding that thapsigargin induces apoptosis is consistent with a recent study showing that thapsigargin activates NADPH oxidase (Granfeldt et al., 2002). It is likely that thapsigargin induces apoptosis in a similar manner to ionomycin, through the induction of oxygen radicals. The normal apoptotic response of MRP-14<sup>−/−</sup> myeloid cells to these Ca<sup>2+</sup> mobilising agents is consistent with the normal Ca<sup>2+</sup> flux and superoxide burst response to these stimuli.

MRP-14<sup>−/−</sup> does not play an essential role in many apoptotic responses of myeloid cells. One pathway that was not examined was Fas/FasL mediated apoptosis, which has been proposed to play a key role in regulating the lifespan of human neutrophils (Ward et al., 1999b). Murine neutrophils express high levels both Fas and FasL. However, my attempts to induce bone marrow myeloid cell apoptosis by antibody crosslinking Fas were unsuccessful. Studies of Fas (lpr) deficient mice suggest that Fas-FasL mediated apoptosis is not important in regulating the lifespan of murine inflammatory neutrophils (Fecho and Cohen, 1998) nor in spontaneous or drug-induced apoptosis (Villunger et al., 2000).
CHAPTER 6

Response of MRP-14<sup>−/−</sup> mice to an inflammatory stimulus <i>in vivo</i>

6.1 Introduction

As <i>in vitro</i> assays never fully reproduce the situation <i>in vivo</i>, the ability of MRP-14<sup>−/−</sup> mice to mount an inflammatory response was tested <i>in vivo</i>. The models of Thioglycollate (TG)-induced peritonitis and <i>Streptococcus pneumoniae</i> (<i>S. pneumoniae</i>)-induced pneumonia were investigated.

When injected into the peritoneum, TG causes the rapid recruitment of neutrophils and monocytes (Henderson et al., 2003). The mechanism by which thioglycollate induces inflammation has not been characterised. However, this is a widely used model to study myeloid cell recruitment to an inflammatory site <i>in vivo</i>.

Pneumococcal pneumonia is a life-threatening illness, and can also lead to systemic bacterial infection and shock. The pathogenesis of murine pulmonary pneumococcal infection has been well characterised (Bergeron et al., 1997). Phagocytosis of <i>S. pneumoniae</i> by resident tissue macrophages and recruited neutrophils is the most important mechanism limiting the bacterial infection in the pre-septicaemic phase. This is an attractive model to study the recruitment of MRP-14<sup>−/−</sup> myeloid cells to a site of infection and their ability to phagocytose and kill bacteria <i>in vivo</i>.
Chapter 6: Response of MRP-14\textsuperscript{+} mice to an inflammatory stimulus \textit{in vivo}

6.2 Results

6.2.1 The response of MRP-14\textsuperscript{+} mice to thioglycollate-induced peritonitis

The ability of MRP-14\textsuperscript{+/−} neutrophils and monocytes to mount an acute inflammatory response was tested \textit{in vivo} using the model of TG-induced peritonitis. TG was injected intraperitoneally, and at selected time points, the elicited leukocytes were harvested, stained and identified by flow cytometry. No significant difference was seen in the rate of either neutrophil (Figure 6.1 A) or monocyte (Figure 6.1 B) influx between MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{+/−} mice. The recruitment of other classes of leukocyte into the peritoneum in response to TG was examined in MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{+/−} mice over a longer time period. No difference was seen between MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{+/−} mice in the numbers of elicited eosinophils, B lymphocytes, CD4 or CD8 T lymphocytes (Figure 6.1 C).

6.2.2 The response of MRP-14\textsuperscript{+} mice to Streptococcus pneumoniae infection

The ability of MRP-14\textsuperscript{−/−} mice to respond to a bacterial infection was tested using the model of \textit{S. pneumoniae}-induced pneumonia. This work was performed by Dr Aras Kadioglu at the University of Leicester. Pneumonia was induced by intra-tracheal installation of \textit{S. pneumoniae} and the disease was monitored over 72 hours by examining the numbers of bacteria recovered from the lungs and blood.
Figure 6.1 Migration of leukocytes into the peritoneum after thioglycollate injection
(A) Neutrophil and (B) monocyte migration into the peritoneum of MRP-14+/+ (filled shapes) and MRP-14−/− (open shapes) mice after thioglycollate injection. Data representative 3 experiments. (C) Migration of various classes of leukocyte into the peritoneum after thioglycollate injection. Data representative of 2 experiments. Data are reported as mean ± standard error of the mean.
The numbers of *S. pneumoniae* recovered from the lungs of MRP-14\(^{+/+}\) and MRP-14\(^{+/−}\) mice were similar (Figure 6.2 A). Over 72 hours the numbers of bacteria approximately doubled. At 24 hours, no bacteria were detected in the blood of MRP-14\(^{+/+}\) mice, but at later time points bacteria disseminated into the blood (Figure 6.2 B). MRP-14\(^{+/−}\) mice were less able to contain the bacterial infection and high levels of bacteria were detected in the blood at 24 hours.
**Figure 6.2 Time course of *S. pneumoniae* infection**

Numbers of bacterial colony forming units (CFU) isolated from the lungs (A) and blood (B) of MRP-14\(^{++}\) (green line) and MRP-14\(^{++}\) (red line) mice following intranasal instillation of *S. pneumoniae*. Data representative of 3 experiments.

Data are reported as mean ± standard error of the mean. *, \( p < 0.05 \)
6.3 Discussion

6.3.1 Normal response of MRP-14\(^{+/+}\) mice to thioglycollate-induced peritonitis.

As in vitro assays never fully reproduce the situation in vivo, it was anticipated that the response to an inflammatory stimulus would reveal a difference between the MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) mice. Surprisingly, there was no difference in the recruitment of MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) neutrophils and monocytes in response to TG-induced peritonitis over a 24 hour time course. This is consistent with the normal migration of MRP-14\(^{+/+}\) myeloid cells seen in vitro, in chemotaxis assays.

Early in the inflammatory response, neutrophils constitute the majority of the infiltrating cells, and despite their low synthetic capacity, are an important source of cytokines (Scapini et al., 2000). In addition, MRP-14 is released from transmigrating neutrophils and is localised on the vascular endothelium (Hogg et al., 1989; Robinson et al., 2002), where it has been proposed to play a role in leukocyte trafficking. In this way, neutrophils that have migrated to an inflammatory site may contribute to the regulation of the immune response and the recruitment of other leukocytes. Therefore the response of MRP-14\(^{+/+}\) mice to TG-induced peritonitis was studied over a 72 hour time course and the recruitment of other immune cells was examined. There was no difference in the recruitment of eosinophils, B lymphocytes, CD4 or CD8 T lymphocytes in MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) mice. In addition there was no difference in the maturation of monocytes into macrophages. In summary MRP-
14 appears dispensible for the recruitment of leukocytes into the peritoneum in response to an inflammatory challenge \textit{in vivo}.

\subsection*{6.3.2 MRP-14$^{-/+}$ mice show a faster transition to systemic pneumococcal infection.}

The first indication that the immune response of MRP-14$^{-/+}$ mice was compromised came from studies of \textit{S. pneumoniae}-induced pneumonia in these mice. The numbers of bacteria isolated from the lungs of MRP-14$^{+/+}$ and MRP-14$^{-/+}$ mice were similar over the 72 hour period of the experiment. However, MRP-14$^{-/+}$ mice were less able to contain the infection and bacteria disseminated more rapidly to the blood. In addition, by 48 hours, the MRP-14$^{-/+}$ mice were physically more ill than MRP-14$^{+/+}$ mice, displaying increased signs of lethargy and reluctance to move (moribund) (Dr Aras Kadioglu, personal communication).

The pathogenesis of pneumococcal infection involves a complex interplay between bacterial virulence factors and the host immune response (reviewed in (Gillespie and Balakrishnan, 2000)). In the murine model of pneumococcal pulmonary infection, distinct stages of the disease have been characterised (Bergeron et al., 1997). In the initial stages of infection over the first 24 hours, there is ineffective bacterial clearance by alveolar macrophages, accompanied by an increase in the inflammatory cytokines, TNF-\(\alpha\) and IL-1, in the bronchoalveolar lavage (BAL) fluid. Neutrophils are rapidly recruited within 2-4 hours into the lung tissue. If phagocytosis of pneumococci by macrophages and neutrophils fails to contain the infection, bacteria enter the bloodstream. As \textit{S. pneumoniae} disseminated more
Chapter 6: Response of MRP-14<sup>+/−</sup> mice to an inflammatory stimulus in vivo

rapidly into the bloodstream of MRP-14<sup>+/−</sup> mice, this suggests that some aspect of myeloid cell recruitment or bacterial killing is defective.

The migration of MRP-14<sup>+/−</sup> myeloid cells into the lungs in response to pneumococcal infection has not yet been studied. The migration of MRP-14<sup>+/−</sup> myeloid cells into the peritoneum in response to TG was normal. However, this is a different inflammatory stimulus and many aspects of leukocyte recruitment in the pulmonary circulation are different from those in the systemic circulation (Mizgerd, 2001). MRP-8/14 has been shown to be localized to the vasculature at inflammatory sites, suggesting that MRP-8/14 may have an extracellular role in influencing leukocyte trafficking (Hogg et al., 1989; Robinson et al., 2002). Therefore it cannot be excluded that pulmonary leukocyte recruitment may be defective in MRP-14<sup>+/−</sup> mice. E-/P-selectin double knock out mice have defective neutrophil recruitment in response to pulmonary *S. pneumoniae* infection at 4 hours (Bullard et al., 1996). Unlike MRP-14<sup>+/−</sup> mice, this resulted in significantly reduced clearance of bacteria from the lungs at subsequent time points.

Although it has been shown that MRP-14<sup>+/−</sup> neutrophils can phagocytose *E. coli* at similar levels to MRP-14<sup>+/+</sup> cells (Meg Mathies, personal communication), intracellular bacterial killing by MRP-14<sup>+/−</sup> myeloid cells was not assessed. Therefore, bacterial killing by MRP-14<sup>+/−</sup> neutrophils should now be investigated.

A decrease in the BAL levels of TNF-α and IL-1 and an increase in the blood levels of TNF-α have been correlated with the transition of presepticaemic to septicaemic infection (Bergeron et al., 1997). As this transition occurs more rapidly
Chapter 6: Response of MRP-14\textsuperscript{−/−} mice to an inflammatory stimulus \textit{in vivo}

in MRP-14\textsuperscript{−/−} mice, it would be of interest to study the kinetics of cytokine production in the BAL fluid and blood in these mice.

6.3.3 An additional line of MRP-14\textsuperscript{−/−} mice

Recent work by Manitz and co-workers reports the characterisation of MRP-14\textsuperscript{−/−} mice (Manitz et al., 2003). The immune response of these MRP-14\textsuperscript{−/−} mice was also assessed using the TG model of peritonitis. Consistent with the findings reported in this chapter, the number and subsets of elicited peritoneal leukocytes in MRP-14\textsuperscript{+/−} and MRP-14\textsuperscript{−/−} mice were similar over a 4 hour time course.

Manitz and co-workers used an additional model of leukocyte emigration to test the function of MRP-14\textsuperscript{+/−} myeloid cells. IL-8 was injected into the skin on the ear and emigration of leukocytes was assessed by immunohistochemistry. IL-8 provoked a similar leukocyte infiltrate in MRP-14\textsuperscript{+/−} and MRP\textsuperscript{−/−} mice (Manitz et al., 2003). The ability of MRP-14\textsuperscript{−/−} mice to respond to an infection \textit{in vivo} was not tested.

6.3.4 Other \textit{in vivo} models of infection

The response to \textit{Leishmania donovani} is now recognised to be dependent on Gr-1\textsuperscript{+} leukocytes, potentially neutrophils (Smelt et al., 2000). Preliminary results show that the early response to \textit{L. donovani} is similar in MRP-14\textsuperscript{+/−} and MRP-14\textsuperscript{−/−} mice (Dr Paul Kaye, personal communication).

To further understand the role of MRP-14 it will be important to investigate other models of infection where the response of MRP-14\textsuperscript{−/−} mice may be
Chapter 6: Response of MRP-14\(^{+/−}\) mice to an inflammatory stimulus \textit{in vivo}

compromised. For example, it is of interest to study pneumonia induced by Gram-negative bacteria, such as \textit{E. coli} and \textit{P. aeruginosa} in MRP-14\(^{+/−}\) mice. In addition, the inflammatory response of MRP-14\(^{+/−}\) mice to \textit{S. pneumoniae} infection could be extended using a peritonitis model. Other models of infection that should be studied include experimental urinary tract infection and colitis. These models will be further discussed in Chapter 7.
CHAPTER 7

General Discussion and Future Directions

7.1 The effect of rMRP-14 in vivo is likely to be due to endotoxin contamination

Previous work by Richard May in this laboratory demonstrated that rMRP-14 is a potent chemoattractant for myeloid cells in vivo using the air pouch model of inflammation (May, 1999). Endotoxin contamination was reduced to pg quantities, suggesting that the effect was due to the S100 protein. rMRP-14 did not directly cause chemotaxis or activation of myeloid cells in vitro. However, rMRP-14 caused a rapid elevation in the levels of cytokines (MIP-2, KC, MCP-1, TNF-α and MIP-1α) in air pouch exudate, suggesting that rMRP-14 causes leukocyte influx via an indirect mechanism. Macrophages were identified as a major source of MIP-2 by immunohistochemistry. The ability of rMRP-14 to stimulate macrophage cytokine production was reproduced in vitro, using RAW 264.7 cells.

As a control for endotoxin involvement, air pouch experiments were performed using antibodies to block rMRP-14 or heat inactivation of rMRP-14. However, these experiments were inconclusive. Therefore as a final control for endotoxin involvement, an air pouch experiment was performed using LPS insensitive C3H-HeJ mice. Surprisingly, rMRP-14 did not produce an influx of cells. Picogram levels of LPS, equivalent to the level of endotoxin contaminaton in rMRP-14, induced similar chemokines to rMRP14 in the air pouch. Therefore it is likely the effect of rMRP-14 in vivo is due to endotoxin contamination.
Chapter 7: General Discussion and Future Directions

The finding that the effect of rMRP-14 is likely to be due to endotoxin contamination raises the possibility that the reported inflammatory effects of other S100 proteins in vivo could also be due to endotoxin. Murine MRP-8 and human S100A12, MRP-8 and MRP-14 have been reported to induce the recruitment of neutrophils and monocytes in vivo, with very similar kinetics to the rMRP-14 induced leukocyte infiltrate in the air pouch (Devery et al., 1994; Ryckman et al., 2003; Yang et al., 2001). In addition, the major cell type responding to human S10012 in vitro (monocytes) does not reflect the major cell type responding in vivo (neutrophils). If endotoxin contamination was responsible for the reported chemotactic effects of MRP-8 in vivo, this would explain why a peptide corresponding to only the hinge region of MRP-8 was equally effective as the full length protein at inducing an inflammatory exudate. Therefore, the effects of murine MRP-8 and human S100A12, MRP-8 and MRP-14 in vivo should be confirmed in C3H-HeJ mice. Their activity should also be shown to be sensitive to protease digestion and insensitive to polymyxin B or neutralising antibodies to CD14.

7.2 Lack of MRP-8 protein in MRP-14−/− myeloid cells

MRP-14−/− mice were previously generated by Richard May in this laboratory (May, 1999). Initial characterisation of the myeloid cells from these mice revealed MRP-8 mRNA, but not protein, was present in these cells. Therefore, the expression of MRP-8 mRNA is independent of MRP-14 expression and it is likely that the expression of MRP-8 protein is post-transcriptionally regulated. This is supported by studies with human MRP-8/14, which demonstrate that MRP-8 in particular is unstable in the absence of its partner (Hunter and Chazin, 1998).
Chapter 7: General Discussion and Future Directions

During embryonic development in MRP-14\textsuperscript{+/+} mice, MRP-8 mRNA is expressed in a large region of maternal decidual tissue surrounding the embryo and MRP-14 mRNA is expressed in a much smaller region that overlaps with only part of the region of MRP-8 expression. Therefore, during development the expression of MRP-8 and MRP-14 mRNA are independently regulated. In MRP-14\textsuperscript{−/−} mice, MRP-8 mRNA was expressed in a much smaller area of decidual tissue than in MRP-14\textsuperscript{+/+} mice. Therefore, in contrast to the situation in the bone marrow, the expression of MRP-8 mRNA is dependent on the presence of MRP-14 and is regulated at the level of transcription.

These findings raise some general questions about S100 proteins. Firstly, what are the mechanisms that regulate the expression of S100 proteins in different tissues? The mechanisms that regulate S100 gene expression may involve transcription, translation, mRNA stability, and protein stability. Secondly, the finding that MRP-8 is expressed in the absence of MRP-14 in maternal decidual tissue suggests that it may be forming homodimers or could be associated with partner other than MRP-14.

### 7.3 Reduced sensitivity of MRP-14\textsuperscript{+/−} neutrophils to suboptimal levels of MIP-2

When Ca\textsuperscript{2+} responses were investigated in MRP-14\textsuperscript{+/+} and MRP-14\textsuperscript{+/−} neutrophils, there was no difference in the response to the formylated peptide, fMLP or the maximal response to the chemokine MIP-2. However, at suboptimal concentrations of MIP-2, MRP-14\textsuperscript{+/−} neutrophils were less responsive.
Further work needs to be done to examine whether MRP-14 plays a similar role in other Ca\(^{2+}\) responses, including those induced by other chemoattractants e.g. KC, PAF and MIP-1\(\alpha\), and by integrin and Fc\(\gamma\)R crosslinking. This work could also be extended by using different techniques to monitor intracellular Ca\(^{2+}\) in neutrophils (for a review see (Hallett et al., 1999)). For example, using new “xt scanning” confocal microscopy techniques, rapid temporal and spatial information on intracellular Ca\(^{2+}\) can be obtained from single neutrophils. This technique could be complemented using other fluorescent probes that report on intracellular free Ca\(^{2+}\) changes in different sub-cellular locations, e.g. at the plasma membrane or within Ca\(^{2+}\) storage organelles.

7.4 Where is the defect in the MIP-2 signalling pathway?

It is unclear where the defect in MIP-2 signalling in MRP-14\(^{-/-}\) neutrophils lies. A similar amount of intracellular Ca\(^{2+}\) was released from stores by thapsigargin in MRP-14\(^{+/+}\) and MRP-14\(^{-/-}\) neutrophils. Therefore the defect in MRP-14\(^{-/-}\) cells is not due to problems with Ca\(^{2+}\) storage. fMLP and MIP-2 signalling pathways share many features in common, but only the MIP-2 pathway is defective in MRP-14\(^{-/-}\) neutrophils. Therefore this is an attractive model to study.

MRP-14 may act directly on the MIP-2 receptor, or alternatively, MRP-14 may be involved in regulating the pathway downstream. MRP-14\(^{+/+}\) mice may have a partial defect in the IP\(_3\) pathway, as fMLP, but not MIP-2, uses Ryanodine-gated receptors in addition to IP\(_3\) receptors to release Ca\(^{2+}\) from stores (Partida-Sanchez et al., 2001). There is increasing evidence that chemokine receptors may activate receptor-specific and G-protein independent signal transduction pathways, although
Chapter 7: General Discussion and Future Directions

the molecular basis has yet to be determined. Therefore it remains a possibility that MRP-14 may be important for regulating an as yet undiscovered specific component of the MIP-2 signalling cascade.

MRP-14 has been identified as the major arachidonic acid binding protein in neutrophils (Kerkhoff et al., 1999a). Therefore it will be of interest to study arachidonic acid metabolism in MRP-14−/− cells. Arachidonic acid has been shown to inhibit the IP3 receptor but not the ryanodine receptor (Striggow and Ehrlich, 1997) and can affect the intracellular Ca2+ concentration in many cell types (Striggow and Ehrlich, 1997). Therefore, alterations in arachidonic acid metabolism or transport in MRP-14−/− cells may explain the defect in MIP-2 signalling and slightly elevated basal levels of intracellular Ca2+ in MRP-14−/− neutrophils.

7.5 MRP-14 is not essential for many myeloid functions in vitro

The reduced ability of MRP-14−/− neutrophils to flux Ca2+ did not impair their ability to respond chemotactically to MIP-2. In addition, the myeloid cell functions of phagocytosis, superoxide burst and apoptosis were unaffected in MRP-14−/− cells. One explanation for these findings is that the levels of agonist required to cause a Ca2+ flux are generally much lower than those needed for other neutrophil functions ((van Eeden et al., 1999); Figure 7.1). For example, activation of neutrophil superoxide burst is a less sensitive assay, and requires a higher concentration of stimulus than the chemotaxis assay, which is less sensitive than Ca2+ mobilisation. Therefore MRP-14 may only be required for myeloid functions stimulated by absolutely minimal levels of agonist.
Figure 7.1 A schematic illustration of concentration-dependent neutrophil functional responses
The chemoattractant fMLP serves as an example to illustrate the concept that neutrophils respond in a graded fashion to increasing concentrations of agonist. This figure is adapted from S.F. van Eeden et al., 1999.
Chapter 7: General Discussion and Future Directions

7.6 A role for MRP-8 and MRP-14 during embryonic development

MRP-14<sup>−/−</sup> mice expressed reduced levels of MRP-8 mRNA in maternal decidual tissue and MRP-14<sup>−/−</sup> embryos developed normally. This is in contrast to MPR-8<sup>−/−</sup> mice that are reported to die in utero (Passey et al., 1999a). Therefore, MRP-8 plays an essential function during development that is independent of MRP-14. In contrast to the findings of Passey et al., MRP-8 mRNA was shown to be expressed by maternal decidual cells surrounding the embryo and not migrated fetal trophoblasts. In addition, I have shown that MRP-8 mRNA is expressed by the majority of cells within the placental labyrinth at 10.5 d.p.c. This provides an explanation of why MRP-8<sup>−/−</sup> embryos born to MRP-8<sup>−/−</sup> mothers die in utero. By using tetraploid aggregation chimeras, it will be possible to prove whether MRP-8 expression is only critically required by fetal cells in the extra embryonic tissues of MRP-8<sup>−/−</sup> mice.

It is known that genes that function in placental development often have other roles in other cell lineages during development or in the adult (Rossant and Cross, 2001). The finding that MRPs are critically involved in development raises the question of whether other S100 proteins have a role in development. This will become clear as the information about other S100 deficient mice becomes available. So far S100A1, S100B, MRP-8 and MRP-14 deficient mice have been reported (Du et al., 2002; Nishiyama et al., 2002; Passey et al., 1999a; Xiong et al., 2000), and of these, MRP-8<sup>−/−</sup> mice are the only ones that display an embryonic lethal phenotype.
7.7 The response of MRP-14"^/ mice to an inflammatory stimulus in vivo

The migration of MRP-14"^/ myeloid cells to an inflammatory stimulus in vivo was investigated using the model of thioglycollate-induced peritonitis. There was no difference in the recruitment of MRP-14"^/+ and MRP-14"^-/ mice of neutrophils and monocytes. The ability of MRP-14"^-/ mice to respond to an infection in vivo was tested using the model of S. pneumoniae-induced pneumonia. MRP-14"^-/ mice were less able to contain the infection and bacteria disseminated more rapidly to the blood than in MRP-14"^/+ mice. These findings should now be extended to examine whether MRP-14"^-/ mice have a defect in myeloid cell recruitment or microbial killing, and then to identify the molecular details of the defect. It has been reported that neutrophils use distinct pathways for emigration into the lungs in response to Gram-positive and Gram-negative pneumonia (Mizgerd et al., 1999). Therefore it would also be interesting to examine the response of MRP-14"^-/ mice to pneumonia induced by Gram-negative bacteria, such as E. coli and P. aeruginosa. MRP-14 may be differentially important for these different classes of bacterial infections.

7.8 Future in vivo studies to investigate the function of MRP-14

Models of infection

MRP-14"^-/ mice demonstrated a reduced ability to contain a pulmonary streptococcal infection in vivo. It will also be important to examine the response of MRP-14"^-/ mice in other models of infection. This may help to elucidate whether the defect in the immune response of MRP-14"^-/ mice is specific to particular pathogens and tissue environments.
Chapter 7: General Discussion and Future Directions

The model of experimental urinary tract infection is an excellent system to study MRP-14<sup>-/-</sup> neutrophil recruitment and microbial killing (Godaly et al., 2001). In this model, bacteria are injected into the bladder. The disease is monitored by studying the recruitment of neutrophils and clearance of bacteria at the infected mucosal site, and by studying the dissemination of bacteria to other tissues.

**Chronic inflammation**

Elevated plasma levels of MRP-8/14 are associated with many chronic inflammatory diseases such as rheumatoid arthritis and sarcoidosis (Bullock et al., 1982). Therefore, it is of interest to determine whether MRP-8/14 contributes to the pathogenesis of such inflammatory lesions using MRP-14<sup>-/-</sup> mice. Collagen-induced arthritis (CIA) is the most widely used murine model of arthritis (reviewed in (Lindqvist et al., 2002)). However, the susceptibility of mice to CIA is critically dependent on the presence of a single MHC class II haplotype, I-A<sup>d</sup>. This model is unsuitable for MRP-14<sup>-/-</sup> mice on a C57BL/6 background as they do not possess this haplotype. However, murine models of colitis are suitable for studying chronic inflammatory disease in MRP-14<sup>-/-</sup> mice. Colitis can be induced by exposure to physical agents, such as dextran sulfate sodium, which appear to disrupt the epithelial barrier and promote cellular exposure to normal mucosal microflora (Strober et al., 2002). Mucosal macrophages are activated and release pro-inflammatory cytokines and initiate of the inflammatory response. Colitis can be scored histologically, by examining mucosal inflammation, and by assessing plasma levels of cytokines, such as TNF-α.
Adaptive immune function

MRP-14 is released from migrating myeloid cells and is localised to the vascular endothelium where it has been suggested to have a role in leukocyte trafficking (Hogg et al., 1989; Robinson et al., 2002). In addition, myeloid cells that are recruited to an inflammatory site release inflammatory mediators that influence the immune response (Scapini et al., 2000). Therefore, the characterisation of the immune function of MRP-14\textsuperscript{-/-} mice should be extended to examine the functioning of the adaptive immune system.

Delayed-type hypersensitivity (DTH) reactions can be used to test antigen uptake and processing by antigen-presenting cells (APCs) and T cell priming and activation in MRP-14\textsuperscript{-/-} mice. In this model, antigen is injected into subcutaneous tissue and is processed by local APCs that migrate to the draining lymph node to prime naïve T\textsubscript{H}1 cells. Subsequent exposure to the antigen \textit{in vitro} or \textit{in vivo} causes a DTH reaction. \textit{In vitro}, cells from the draining lymph node are cultured with antigen. APCs process and present the antigen to effector T\textsubscript{H}1 cells, which are stimulated to proliferate. Initial studies with MRP-14\textsuperscript{-/-} mice reveal no difference in the proliferative responses of T cells compared to MRP-14\textsuperscript{+/+} mice (Eileen McNeill, personal communication). The DTH reaction should also be studied \textit{in vivo} in MRP-14\textsuperscript{-/-} mice. After rechallenge with antigen, a local allergic reaction occurs, accompanied by an inflammatory cell infiltrate and oedema. The inflammatory response can be scored clinically by measuring tissue swelling, or histologically by examining the extent of leukocyte infiltration.
Chapter 7: General Discussion and Future Directions

Epithelial cell function

Murine MRP-8 and MRP-14 have been shown to be expressed by murine keratinocytes and myeloid cells in vivo during skin inflammation, wounding and all stages of carcinogenesis (Gebhardt et al., 2002; Thorey et al., 2001). Therefore, it is of interest to determine whether MRP-8/14 contributes to the pathogenesis of such conditions. This could be studied using the model of phorbol ester (TPA)-induced skin inflammation and carcinogenesis (as described in (Gebhardt et al., 2002)). Acute application of TPA causes skin inflammation. Chronic application of TPA can be used to study the development of skin dysplasia, papillomas and squamous cell carcinomas in MRP-14\(^{-/-}\) mice.
References


References


References


**References**


References


References


References


References


References


References


233
References


Peters, L. L. and Barker, J. E. Percentages of peripheral blood leukocyte subsets in various inbred mouse strains: Mouse Phenome Database.


References


binding proteins MRP8 (S100A8) and MRP14 (S100A9) binds unsaturated fatty acids with high affinity. *J Biol Chem* **272**, 9371-7.


References


differentiation, modulates Ca2+-dependent translocation from cytoplasm to membranes and cytoskeleton. *J. Immunol.* **156**, 1247-1254.


Publications arising from this thesis
