An investigation into the function of two murine S100 proteins, MRP-8 and MRP-14

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Abstract

Neutrophils are the fastest responding leukocytes in an inflammatory response. Two major components (up to 45%) of the neutrophil’s cytosolic protein are MRP-8 and MRP-14. MRP-8 and MRP-14 are members of the S100 family of calcium binding proteins, designated MRP-8 and MRP-14, respectively. They have tissue specific expression and are found in myeloid cells and specialised epithelium.

Previous work from this laboratory suggests a potential role for the proteins in inflammation. Investigation of recombinant human MRP-8 and MRP-14 (rHuMRP-8 or 14) showed rHuMRP-14 to be a novel activator of the β2 integrin, Mac-1 on neutrophils, causing ligation of fibrinogen. rHuMRP-8 inhibited this stimulated adhesion by formation of a rHuMRP-8/14 heterodimer.

This thesis aims to further investigate the function of these enigmatic proteins by analysing the murine homologues, also known as MRP-8 and MRP-14. rMuMRP-8 is reported to be a potent (10^{-12} M) neutrophil and monocyte chemoattractant. rMuMRP-14 was found to share functional similarity with rHuMRP-14 by causing neutrophil adhesion without causing chemotaxis or respiratory burst. rMuMRP-14 was also found to be functionally dissimilar to rHuMRP-14 by causing cell activation of resting peripheral cells, but not activated, pre-migrated cells, as measured by intracellular calcium flux. In vivo, in an air pouch model of inflammation, rMuMRP-14 caused profound neutrophil and monocyte infiltration with a peak response at 6 hours with a 50 μg dosage. Taken together, these data suggest that rMuMRP-14 is a direct activator of cell adhesion but an indirect activator of cell migration.

Protein investigation was further investigated by the creation of knockout mice. Both muMRP-8 and muMRP-14 deficient animals were healthy and fertile with grossly normal neutrophils. The mice await detailed phenotyping.

In summary, muMRP-8 and muMRP-14 have functional extracellular roles in inflammation but their absence affects neither mouse viability nor gross neutrophil morphology.
Dedication

This thesis is dedicated to the four people who kept me going when most of the lights went out and who enabled me to attempt to scale the mountain range of science. To my parents, Paula and Adrian Williams and to my Uncle and Aunt, Pam and Alan Jenkinson.
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1. Introduction

The immune system has evolved to protect the body from invading pathogens. Generally speaking, there are two main types of immunity, innate and adaptive. Innate immunity is always present, directed against generic antigens such as bacterial peptides. In contrast, adaptive immunity is specific to and driven by foreign antigens of any chemical composition and results in immunological memory. However, the two arms of the immune system do integrate in order to resolve an infection.

In the case of a viral infection, the virus infects a tissue dendritic cell which synthesises peptides derived from viral proteins and presents these proteins, in association with MHC class I or class II molecules on the cell surface. At this stage in its life, the dendritic cell is highly phagocytic and weakly co-stimulatory for lymphocytes. The effect of the viral infection on the body causes the dendritic cell to leave the tissue and migrate to a lymph node during which time it matures, losing its phagocytic capabilities and increasing the level of MHC class II and co-stimulatory molecules such as B7, on its surface. If antigen is presented to a T lymphocyte in association with MHC class I and co-stimulatory molecules, then a CD8 (cytotoxic) response is generated, whereas if antigen is presented in association with MHC class II and co-stimulatory molecules, then a CD4 (helper) response is generated. Whichever subclass of lymphocyte responds, the next step is clonal expansion, which occurs in the lymphoid organ. The responsive T-cell leaves the lymphoid organ and enters the circulation. The time from antigen presentation to production of effector cells is approximately 5 days.

Activated T cells can then activate B-cells that are also specific for the virus. Antigen specific B cells bind antigen via their cell surface immunoglobulin receptors and internalise it, before digesting it into peptides and presenting it in association with MHC class II molecules on the cell surface. The presented antigen-MHC class II complex is then recognised by a virus specific T helper cell which activates the B cell to proliferate. Proliferation occurs in the germinal centres of the lymph node and results in the production of effector B cells that can secrete antibody against the pathogen.

Virally-infected cells with viral products displayed in their cell membrane are then coated with antibody which marks the cell for attack by macrophages. Other infected cells that display viral antigens in association with MHC molecules, and also express a co-stimulatory molecule, are recognised by the CD4 or CD8 T-lymphocyte
are then indirectly or directly attacked, respectively. In this way, the infection is
resolved.

The innate immune system does not adapt. Its receptors have evolved to
recognise general pathogens, such as bacteria. It protects the body in the early phase of
infection whilst a specific response is being mounted and it is utilised as an effector in
the later stages of the specific immune response. The main mediators of the innate
response are the complement system, the acute phase proteins and the myeloid cells.
The myeloid cells develop in the bone marrow and are derived from a common myeloid
progenitor. This class consists of neutrophils, monocytes (and from monocytes,
macrophages), basophils and eosinophils.

Neutrophils, monocytes and macrophages directly kill bacteria by phagocytosis.
Phagocytosis can be via complement recognition or opsonisation of a bacterium by
antibody. The engulfed bacteria are taken into a phagosome which is then fused with
various granules containing antibacterial proteins and others filled with superoxide
radicals, generated from the NADPH-oxidase system.

A new view of immune function also presents evidence for substantial
integration of the innate and adaptive arms of the immune system. The only way that
the innate system can be effective is to recognise vital base features of pathogens that do
not change, such as LPS from gram negative bacteria. Pathogen infection results in
recognition by and activation of the innate immune system via production of Toll-
receptor ligands. The Toll-receptor ligand then activates the transcription factor
NFκB and hence production of co-stimulatory molecules such as B7.1 (178). This then
allows activation of the adaptive immune system.

The vascular system is a transport system allowing transit of essential nutrients
and cells throughout the body. As infection is often found in the tissues, the cells of the
immune system must leave the blood at a convenient site and migrate into the tissue to
mediate their beneficial effects. The mechanism by which this is achieved is described in
the next section.

The section above was written with reference to the following (16, 118, 177).
Chapter 1: Introduction

1.1 Neutrophils and their role in the immune response and inflammation

This thesis is concerned with the function of 2 intracellular proteins found mainly in neutrophils and monocytes. Therefore, this section of the introduction will deal with myeloid cell trafficking and recruitment rather than lymphocyte trafficking and recruitment.

1.1.1 Adhesion cascade - rolling, activation, firm adhesion and diapedesis

Leukocytes are a class of “white blood cells” involved in immune function. Members of this class of cells are lymphocytes, neutrophils, monocytes, basophils and eosinophils. The interactions described below for neutrophil-endothelium and neutrophil-epithelium are generally applicable to the other leukocytes.

The adhesion cascade is a four step reaction characterised by cell rolling, cell activation, firm adhesion and finally diapedesis (transmigration). This process involves several important classes of adhesion molecules including the selectins, integrins and the immunoglobulin superfamily. The steps in this model are sequential and one does not occur without the other preceding steps.

Before describing the paradigm of neutrophil migration, a brief introduction to the classes of adhesion molecules involved in the process will be made. The selectins are lectin molecules with a Ca\(^{2+}\)-dependent lectin-homologous N-terminal domain, an epidermal growth factor like domain and between 2 and 9 repeat domains that are homologous to domains found in complement binding proteins (21, 219). There are various forms of selectin molecules, P-selectin (on endothelial cells and platelets), E-selectin (vascular endothelial cells) and L-selectin (leukocytes) (244). All circulating leukocytes surface express L-selectin, except some memory lymphocytes (244). The ligands for selectin molecules are mucins. Specifically, selectins recognise a sialylated carbohydrate on their ligands, generally derived from sialyl Lewis\(^x\) and Lewis\(^a\) (219).

Integrins are transmembrane glycoproteins comprised of an \(\alpha\) (120-180kDa) and \(\beta\) (90-110kDa) subunit which non-covalently associate into a heterodimer (111). The name, “integrin” refers to the function of this family of molecules which is to integrate the intracellular cytoskeleton with the extracellular matrix (110). Integrins generally recognise more than one ligand; indeed, ligands are generally recognised by more than one integrin (111). There are more than 22 integrins (249) and with the exception of erythrocytes, all cells possess integrins (95).
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The immunoglobulin superfamily (IgSF) have repeated immunoglobulin-like domains (90-100 amino acids) which consist of 2 β-sheets, held together with a disulphide bridge (reviewed in (76)). Members of this family include a subset involved in adhesion reactions such as ICAM-1 to -3, identified through their binding to LFA-1 (243) and VCAM-1. ICAM-1 has 5, ICAM-2 has 2 and ICAM-3 has 5 repeated immunoglobulin-like domains. ICAM proteins are heavily glycosylated, type-1 membrane glycoproteins with one membrane spanning domain.

A generalised scheme of transendothelial migration is shown in Figure 1.1A (page 29). The first step in transendothelial migration is rolling, a reversible process shown to be mediated by L-selectin (264). On neutrophils, L-selectin is highly expressed, constitutively active and mediates rolling on activated endothelium; that is, L-selectin binding is active although the neutrophil itself has not been activated. This neutrophil rolling slows the cell down and is thought to permit reactivity with local chemoattractants or other activating signals (39). The ligands for L-selectin are GlyCAM-1 (146) and podocalyxin (226) (on high endothelial venules) and CD34 (on normal endothelium) (17). E- (149) and P-selectin molecules (175), present on endothelium can also be involved in mediating neutrophil rolling. P-selectin mediates this reaction via interaction with its ligand, PSGL-1 (187) whereas E-selectin can mediate its interaction through various ligands, including sialyl-Lewis X (a motif on all selectin ligands) (206) and PSGL-1 (11). After cellular activation by thrombin (252) or IL-3 (124), P-selectin can be mobilised to the surface of the endothelium and platelets from intracellular vesicles and then mediate adhesion of myeloid cells and a sub-population of memory T-cells (78). E-selectin is primarily endothelially expressed after activation by cytokines and LPS but also mediates the adhesion of myeloid cells and a sub-population of memory T-cells (20).
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B. Stylised general structure of 3 exon S100 family genes. Only exons 2 and 3 are protein coding, the translational start site is shown in exon 2.
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The second step in the cascade is cellular activation. If a neutrophil is activated by a chemoattractant or signal from the endothelium, then cell polarisation (shape change), L-selectin shedding (126) and redistribution of the selectin ligand, PSGL-1, to the uropod occur (162). Also, the amount of surface Mac-1 increases by ten fold (reviewed in (243)) although only 10% is capable of mediating adhesive reactions (58).

The third step is firm adhesion to the endothelium. This requires integrin activation, which is a transient event (243). This transience then allows de-adhesion of the trailing edge of a cell in a locomotion (244). Leukocyte integrins do not mediate ligand binding reactions unless they are activated (148). As shown for LFA-1, with monoclonal activating antibodies such as 24, (142) integrin activation results in a conformational change in the molecule. LFA-1 activation and hence binding is generally thought to involve two processes of increases in avidity (integrin clustering) and affinity (conformation changes causing increased strength of individual interactions) (250) (reviewed in (248)).

Various factors can activate integrins to bind ligand. As with step 2, the activation step, chemoattractants such as fMLP and IL-8 can activate integrins (145). In neutrophils, firm adhesion involves the β2-integrins, LFA-1 and Mac-1 binding to the endothelial ligand ICAM-1 (60, 61, 240), although both LFA-1 (246) and Mac-1 (274) also bind the constitutively expressed ICAM-2. Also, Mac-1 binds to fibrinogen and fibrinogen can bind ICAM-1 (144). ICAM-1 endothelial expression can be up-regulated by various inflammatory mediators including IFN-γ, TNF-α and IL-1 (199). A recent report has shown that the initial 30 seconds of neutrophil firm adhesion to ICAM-1 is mediated by both LFA-1 and Mac-1 whereas later adhesion, up to 2 minutes, is mediated by Mac-1 (195).

Finally, again using the β2-integrins LFA-1 and Mac-1, the neutrophil extravasates through the endothelium and migrates towards a chemotactic stimulus, in the tissues. Fibrinogen is a facilitator of transendothelial leukocyte migration via its interactions with ICAM-1 (143). A recent study has shown that neutrophil transendothelial migration is LFA-1 and Mac-1 dependent and that the ligands involved include ICAM-1/ICAM-2 but also some, as yet, unidentified ligands (116). The actual site of neutrophil transendothelial migration has been identified as the tri-cellular corners, formed by the junction of three endothelial cells (37).
Integrin-associated protein (IAP or CD47), a 60kDa, IgSF family member expressed on all cell types also has a role in transendothelial migration (49). CD47 directly associates with $\alpha_6\beta_3$ (158) and antibodies to CD47 inhibit $\alpha_6\beta_3$ binding to vitronectin (159). It is though possible that CD47 plays a role in de-adhesion rather than adhesion of cells (203).

Other leukocytes use slightly different sets of adhesion molecule and chemoattractant / chemoattractant receptor interactions when moving from the vasculature to the tissues. This means that great specificity in cellular recognition can occur, by the selective expression of adhesion molecules and activating factors on the endothelium (39). The most important feature of this is the activation of the adhesion receptors, without which an interaction will not occur (111). This is demonstrated physiologically from the observation that although high endothelial venules express L-selectin ligands which mediate neutrophil and lymphocyte rolling, only lymphocytes extravasate into the lymph nodes (reviewed in (120)). One of the reasons for this differential response is the type of chemokines expressed on the endothelial surface. For example, B cells are almost exclusively recruited to secondary lymphoid organs by the B-lymphocyte chemoattractant (BLC) acting on the B cell receptor, Burkitt's lymphoma receptor 1 (BLR-1) (88). In contrast, naïve T cells are recruited by secondary lymphoid-tissue chemokine (SLC) (89) which acts via the CCR7 receptor (271).

As mentioned above, great diversity in response can also be generated by the subtypes of chemokine receptors expressed on cells, as most chemokines bind more than one receptor and most receptors bind more than one chemokine. Considering monocytes (which express CCR1,2 and 5 and CXCR4) and neutrophils (which express CXCR1,2 and 4), both cell types respond to SDF-1 via CXCR4 but their other chemokine receptors render monocytes responsive to various chemokines including RANTES and the MCP family whereas, in contrast, neutrophils are responsive to various chemokines, including IL-8 (reviewed in (13)).

In neutrophil transepithelial migration, a slightly different set of adhesion molecules to the set involved in transendothelial migration are used. There does not appear to be a role for L-, E- or P- selectin in transepithelial migration although other carbohydrate molecules may be involved (47). Transepithelial migration is paracellular, resulting in the reversible disruption of epithelial tight junctions (194). Mac-1 is the
most important integrin for transepithelial migration (202) although its epithelial ligand is not known, none of the known Mac-1 ligands including fibrinogen or ICAM-1 are involved (201). ICAM-1 is actually located on the apical or luminal surface of the epithelium (202). This localisation might have the effect of retaining cells at the site of inflammation under flow conditions (201). CD47 is also important in transepithelial migration (203).

There are various types of chemoattractants that may act to recruit neutrophils from the vasculature to the tissues. These include classical chemoattractants, such as the bacterial product fMLP, the mast cell product histamine, activated complement factor C5a and the chemokine family. The chemokines are approximately 10kDa proteins with 4 conserved cysteine residues which form 2 disulphide bonds (Cys1-Cys3 and Cys2-Cys4) (13). The chemokine family is divided into the CC and CXC sub-families according to whether the first 2 cysteines are adjacent or separated, respectively. Chemokines mediate their effects through G-protein coupled 7 transmembrane spanning receptors which are subdivided into two subfamilies, the CCRs and the CXCRs. In general, CXC chemokines, such as IL-8, acting through CXCR1/2 are neutrophil chemoattractants whereas the CC family, such as RANTES, acting through CCR1/3/4/5 are monocyte and lymphocyte chemoattractants (13). IL-8 is thought to cause transendothelial migration \textit{in vivo} by the formation of a chemokine gradient, as mimicked by \textit{in vitro} chemotaxis assays (109). The chemokines are able to induce neutrophil effector functions other than chemotaxis (14). This contrasts with other "pure" chemoattractants such as substance P and TGF-β, so called because they can cause chemotaxis without activation (90).

When neutrophils are making the transition from the circulation across the endothelium a chemoattractant would be optimal if it could attract the cells without activating their effector functions, such as the superoxide burst. In contrast, activation of effector functions may well be optimal in the tissues. The idea of a chemoattractant gradient then develops new significance. Evidence for this differential function includes such data as IL-8 promoting neutrophil chemotaxis at $10^{-7}$M to $10^{-8}$M but promoting granule release at $10^{-6}$M (234).

This section has attempted to introduce the basics of neutrophil migration and some of the adhesion molecules and chemoattractants that are involved in this process, \textit{in vivo}. The laboratory has been interested for some time in two proteins that are very
highly expressed in neutrophils. These proteins are members of the S100 family of calcium binding proteins, called MRP-8 and MRP-14, whose role in neutrophil function is only beginning to be elucidated. The next section aims to introduce the S100 family and particularly MRP-8 and MRP-14.

The following reviews were used in the writing of the above section (13, 39, 59, 75, 111, 197, 201, 244, 248, 249)

1.2 The S100 family of calcium-binding proteins

It is well recognised that calcium is an important metal ion, involved in various biological processes including blood coagulation, cell signalling, bone formation et cetera. Calcium binding proteins have evolved to transduce calcium signals or to buffer calcium both intra- and extracellularly. One calcium binding protein family is the EF-hand family. EF hands were named after the high affinity calcium binding E- and F- helices of parvalbumin (94, 133). A subfamily of the EF-hand family of proteins is the S100 protein family. S100 proteins were originally purified from neural tissue as two (a and b) acidic, low molecular mass (approximately 10kDa) proteins which contained two EF hands and formed homo and hetero dimers (185). The name S100 was coined because the proteins were soluble in 100% saturated ammonium sulphate at neutral pH (186). There are now 18 members of the S100 protein family (218) although the following section will concentrate on the function of three S100 protein family members called MRP-8, MRP-14 and the recently discovered, MRP-6.

1.2.1 Discovery and naming of the S100 family members, MRP-8 and MRP-14

MRP-6, MRP-8 and MRP-14 are intracellular calcium-binding proteins of the S100 family. MRP-8 and MRP-14 were originally partially purified as a supposed (MRP-8)/14 heterodimeric complex of 36.5kDa, called the L1 antigen; MRP-8 and MRP-14 were referred to as L1 light and L1 heavy chains, respectively (52). MRP-8 was first cloned in 1987 as the cystic fibrosis antigen (CFAg) gene (64). The first complete isolation of MRP-8 and MRP-14 protein was achieved after purification with a macrophage migration inhibitory factor (MIF) antibody, 1C5 (1C5 is described in (36)) (200). This isolation produced the name commonly used today, MRP (MIF related
protein); The numbers, 8 and 14, refer to the proteins' apparent molecular mass by SDS-PAGE.

As can be seen, the naming of MRP-8 and MRP-14 was initially confusing. Later work linked the original L1 antigen to the CFAg and hence MRP-8 and MRP-14 (8). MRP-8 and MRP-14 have also been known as calgranulin A and B, respectively (270), whereas the MRP-8/14 complex is still known as calprotectin in some circles (247). In an attempt to simplify S100 biology, a new naming system was recently proposed (229) in which members of the S100 family were given S100A# designations, based on their relative location within chromosome 1q21. Under this system, MRP-8 and MRP-14 are known as S100A8 and S100A9, respectively. However, the naming of the molecules as MRP-8 and MRP-14 is the one still most generally used in the literature and hence is the one that will be used throughout this thesis.

1.2.2 Genomic configuration of MRP-8 and MRP-14

Human MRP-8 was first cloned as a cDNA and mapped to chromosome 1 as part of a cystic fibrosis investigation (64). Another isolation of the human MRP-8 cDNA and also the MRP-14 cDNA was reported soon afterwards (200). The human genes for MRP-8 (huMRP-8) and MRP-14 (huMRP-14) have been cloned (139) and are single copy with a three exon structure. As with other S100 genes, only exons 2 and 3 are protein coding (see Figure 1.1B, page 29). The human MRP-8 and MRP-14 genes produce proteins of 93 and 114 residues, respectively.

The human gene contains normal GT-AG splice donor and acceptor sites and also normal TATA- and CAAT- boxes, upstream of the transcriptional start site (139). As with all S100 protein genes so far analysed (293), MRP-8 and MRP-14 have the 12 base pair (bp) S100 protein element (SPE) close to the TATA box, in the 5'-portion of the gene (4). This takes the following sequence (tataaaGGAGCT_GCCTC) in the human MRP-8 gene and (AGGAGCT_GCCTataaa) in the human MRP-14 gene (4), where the lower case sequence is the TATA box, the upper case sequence the SPE and the dash, a gap in the sequence.

The genes for 13 S100 proteins, including MRP-8 and MRP-14 have since been localised to chromosome 1q12-q21 and the murine MRP-8 and MRP-14 genes are found on mouse chromosome 3 (63, 218). The distance between the human MRP-8 and MRP-14 genes is thought to be less than 45kb (218).
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The mouse MRP-8 gene has also been cloned (191). It is similar to the human gene in that it is single copy, has three exons, the first exon is non-coding and the transcriptional start site is in exon 2. Data about the murine MRP-14 gene have not been reported although data presented in this thesis shows that the mouse MRP-14 gene is also organisationally similar to its human counterpart (section 5.3.5, page 218). The murine and human MRP-14 genes do differ as the mouse MRP-14 gene has only one start site (211) whereas the human MRP-14 gene has an alternative start site, 4 codons from the normal start site (67, 255, 262).

The mouse proteins are slightly different in size from the human proteins, being 89 and 113 residues, respectively. In both the human and mouse genes, the intron between exon 2 and 3 interrupts the variable hinge region coding sequence. It has been speculated that this may have contributed to the variability in the hinge region through evolution (139, 191).

In common with the human gene, the mouse MRP-8 gene also contains normal GT-AG splice donor and acceptor sites and normal TATA- and CAAT- boxes, upstream of the transcriptional start site (191). Also, a 64% nucleotide homology was found in the 400bp upstream of the TATA box, between the mouse and human MRP-8 gene; this is as high as the homology in the coding regions. It is thought that this homology might suggest a conserved transcriptional machinery.

1.2.3 MRP-8 and MRP-14 gene and protein expression

The majority of MRP-8 and MRP-14 gene and protein expression studies have been human. Therefore, the human and mouse data will be discussed separately, with the human data discussed first.

1.2.3.1 Human data

MRP-8 and MRP-14 proteins are highly expressed in cells of myeloid origin, particularly neutrophils and monocytes (200, 296). Macrophages do not normally express MRP-8 or MRP-14 but MRP-14 is expressed in acute inflammatory conditions such as psoriasis and both MRP-8 and MRP-14 are expressed in chronic inflammatory states such as primary chronic polyarthritis (32, 200) and Crohn’s disease (165).

Subcellularly, MRP-8/14 are distributed mainly in the cytosol although they are not stored in granules or endosomes (67). One report suggests that the proteins are
present in the nucleus (189). MRP-8 and MRP-14 can represent approximately 1% of
monocyte and 45% of neutrophil, total cytosolic protein; this represents a 3mM
intracellular concentration in neutrophils (67). Densitometry analysis has suggested that
MRP-8 represents 19% and MRP-14 30% of total neutrophil cytosolic protein (86).

Other tissues expressing MRP-8 and MRP-14 include human mucosal squamous
epithelia (pharynx, vagina/cervix and oesophagus) but not general epithelia (lung, liver,
kidney) (28), normal skin or secretory epithelia (27).

MRP-8/14 expression has been correlated with various disease states.
Expression has been detected in various skin disorders including psoriasis, lichen planus,
and atopic dermatitis (74, 123, 135) whereas healthy skin is negative (27). MRP-14 and
MRP-8/14 complex are also present on the reactive microglia present in Alzheimer’s
disease (3) and cerebral malaria (230) but not on the microglia from the undiseased CNS.

Expression of MRP-8 and MRP-14 has been proposed to distinguish between
immunological and non-immunological granulomas (54). Mononuclear phagocytes from
foreign body granulomas express both MRP-8 and MRP-14 whereas MRP-14 but only
weak MRP-8 expression is noted in the mononuclear phagocytes from tuberculosis.
The main difference between the 2 types of granuloma is that the second is an immune,
T-cell mediated granuloma.

Interestingly, keratinocyte MRP-8/14 expression can be induced if epidermis is
cultured for 24 hours. MRP-8/14 was produced, de novo, and was associated with the
cytoskeleton (43) in a calcium dependent manner (122). It has been shown that
tazarotene, a retinoic acid receptor specific retinoid, which is effective in treating
psoriasis, down regulates the abberant MRP-8 expression seen in psoriatic
keratinocytes (193).

High serum levels of protein have been found in chronic lung disease associated with
cystic fibrosis (34), chronic obstructive pulmonary disease (161), rheumatoid arthritis
(31, 32) and Crohn’s disease (165). MRP-8 was originally thought to be the cystic
fibrosis antigen (CFAg) (64) but later reports suggested that MRP-14 or the MRP-8/14
complex may be the CFAg (30). This situation was resolved when the MRP-8/14
complex was shown to be the CFAg (270). Later studies showed that MRP-8/14
mRNA along with CFTR mRNA were upregulated in the cultured tracheal gland cells of
cystic fibrosis patients (216). This lead to the proposition that in cystic fibrosis, gene
regulation of MRP-8/14 and CFTR are related.
1.2.3.2 Mouse data

In the mouse, the major site of MRP-8 and MRP-14 gene expression is the bone marrow, where 50% of the cells are both MRP-8 and MRP-14 positive; these cells are neutrophils and to a lesser extent monocytes (80). Additional expression sites include the spleen and lung (presumably from circulation-derived myeloid cells) (113, 140). Within the spleen, MRP-8/14 positive cells are found, along with Mac-1 positive and Gr-1 positive cells (myeloid markers) in the marginal zone and red pulp (140).

Gene expression has also been studied during development. Murine MRP-8/14 positive cells were detected from embryonic day 11 in the yolk sac and liver (140). Human MRP-8/14 positive foetal tissues included skin hair follicles, tongue and oesophagus (270).

Murine MRP-8 and MRP-14 have also been found in several models of inflammation such as contact dermatitis (222), cutaneous leishmaniasis (254) and corneal angiogenesis (253), confirming peripheral myeloid cell expression.

1.2.4 Biochemistry and function of S100 proteins

Structural analysis suggests that the S100 proteins do not act as buffers within cells but molecular switches (228). As the conformation of S100 proteins changes when calcium is bound, it is thought that these calcium bound forms may then affect intracellular processes. Indeed, the S100 family are thought to be involved in cell differentiation, cell cycle progression, protein phosphorylation regulation and membrane-cytoskeleton interactions (128).

Within the S100 family, the N terminus is conserved whereas the C terminus is variable. Family members also have two conserved, calcium-binding EF hands (alpha helix, loop, alpha helix). In human MRP-8 and MRP-14, the N-terminal EF hand is a variant EF hand of 30 residues whereas the C-terminal, normal EF hand contains 28 residues (139) (see Figure 1.2A, page 41). The C-terminal hand has a higher affinity for calcium than the N-terminal hand (128). In between the two hands is a variable hinge region. It has been postulated that upon binding of calcium, protein conformation alters, exposing the variable hinge region and thereby generating functional specificity (128). None of the family has a signal sequence although it is thought a conserved region at the N-terminus may act as an internal signal sequence (128).
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The majority of S100 proteins are not leukocyte proteins and so far have not been reported to affect leukocytes. Therefore, they will not be discussed in detail. However, two S100 proteins, psoriasin (S100A7) and S100L have been found to be chemotactic for leukocytes.

Bovine S100L was isolated from lung and found to be chemotactic for eosinophils at 0.1nM (131). S100L was not chemotactic for neutrophils or monocytes. Two forms of the S100L receptor were identified and the associated signal transduction from both isoforms found to be pertussis toxin sensitive.

Human psoriasin (S100A7) is named because of its original detection in psoriatic keratinocytes (168). Psoriasin is chemotactic for both CD4+ T cells and neutrophils at $10^{-11}$M (119). The bovine homologue of psoriasin, CAAF2, has been identified and cloned from amniotic fluid (100). As was found with psoriasin, CAAF2 was expressed in epidermal keratinocytes but it was also found at high levels in amniotic fluid. Chemotactic abilities were not profiled in this study.

1.2.5 Biochemistry and functions of the MRP proteins

As this section will illustrate, although the mouse and human MRP-8 and MRP-14 proteins are 59% homologous at the protein level (140) (see Figure 1.2, page 41), their functions can be quite different. Therefore, functions ascribed to the human proteins will be described first followed by functions ascribed to the mouse proteins.

1.2.5.1 Human MRP-8 and MRP-14

1.2.5.2 MRP-8/14 complex

The fact that MRP-8 and MRP-14 form various complexes has been known since their original isolation (200). It has been suggested that in vivo MRP-8 and MRP-14 form calcium-dependent, non-covalently linked MRP-8/14 heterodimers, heterotrimers (MRP-8)$_2$/14 and heterotetramers (MRP-8)$_2$/((MRP-14)$_2$) but that covalently linked homo- and hetero- dimers do not form (255). A contrasting report suggested that although MRP-8 and MRP-14 predominantly form the non-covalently associated MRP-8/14 heterodimer, some covalently associated MRP-8/14 complex does exist (67). Interestingly, most suggestive functional data is associated with the MRP-8/14 complex although there are notable exceptions (79, 198).
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The heterodimeric MRP-8/14 complex has also been found adjacent to migrating myeloid cells, on the surface of vascular endothelium (103) (see Figure 1.2B, page 41). MRP-8/14 complex has also been found at the site of interaction between the onchocerciasis parasite and infiltrating macrophages (68). These observations suggested that the protein complex may have a role in cell migration to sites of inflammation. MRP-8 and MRP-14 may also be involved in gene transcription. The MRP-8/14 complex was shown to inhibit casein kinase I and II (Kι, \( \leq 1\mu m \)) (189). These kinases are involved in the stimulation of transcription, by phosphorylation, of RNA polymerase and topoisomerase. The inhibition of casein kinase I and II was specific as it did not involve inhibition of various other kinases. It was subsequently hypothesised that MRP-8/14 may be acting to prevent gene transcription and by this mechanism regulate the terminal differentiation of neutrophils (97).

MRP-8 and MRP-14 act as differentiation markers, appearing at a late stage in myelomonocytic differentiation. The leukaemic HL60 cell line (expresses both MRP-8 and MRP-14 mRNA) can differentiate into either monocytes or granulocytes. Induced differentiation down the monocytic pathway with PMA caused loss of MRP-8/14 RNA whereas differentiation down the granulocytic pathway with DMSO caused increased message expression (139). A later study showed that monocytic differentiation with vitamin D3 upregulated MRP-8/14 expression whereas TNF-\( \alpha \) and IFN-\( \gamma \) did not, demonstrating the existence of different monocytic differentiation pathways (221).

1.2.5.3 Subcellular localisation and translocation

The heterodimeric MRP-8/14 complex is present in the membrane of activated monocytes / macrophages, as demonstrated by binding of the monoclonal antibody, 27E10 (22). Cell surface expression of this epitope was upregulated by LPS, PMA and IFN-\( \gamma \) (297), by increases in intracellular calcium levels (22) and also by cell adhesion (169). The functional consequence of this membrane expression is as yet unclear. Upon neutrophil (151) and monocyte activation (22), MRP-8 and MRP-14 undergo translocation from the cytoplasm to the membrane, in a calcium-dependent fashion. Moreover, in monocytes, the MRP-8/14 complex, MRP-8 and MRP-14, associate with vimentin intermediate filaments and the cell membrane after treatment with calcium ionophore (38, 220). Under similar conditions in human squamous carcinoma epithelial
cells, the proteins were found to be associated with keratin intermediate filaments (81). The functional consequence of these translocations is also as yet unclear.

1.2.5.4 Protein phosphorylation

Both MRP-8 and MRP-14 can be phosphorylated which may then regulate their function. Threonine residue 113 in MRP-14 can be phosphorylated after calcium flux (66) but not after PKC activation. In slight contrast, another report suggests that fMLP, PMA (which directly activates PKC) and calcium ionophore A23187 can phosphorylate MRP-14 (18).

Both full-length and truncated forms of phosphorylated MRP-14 are preferentially translocated from the cytoplasm to the membrane after calcium influx; this was associated with increased calcium binding by the phosphorylated as compared to the non-phosphorylated forms of MRP-14 (262). Mouse MRP-14 is not homologous in this region and does not contain a threonine residue (140).

MRP-8 can also be phosphorylated, but at an unknown residue, after stimulation with PMA but not calcium ionophore (87). MRP-8 phosphorylation was blocked by PKC inhibitors. Phosphorylated MRP-8 was restricted to the cytosol and, in contrast to previous findings with MRP-14 (262), phosphorylation inhibited the calcium-dependent translocation of MRP-8 from the cytosol to the membrane.
Figure 1.2: A. Amino acid sequence of murine and human MRP-8 and MRP-14. Structural motifs such as a-helix turns, calcium binding loops and zinc binding regions are shown in blue, red and green, respectively. Translational start sites are underlined and bolded. This figure is based on information published in (140) and (211). B. Immunoperoxidase staining of an area of inflamed rheumatoid synovium labelled with the anti MRP-8/14 monoclonal antibody, 5.5 showing MRP-8/14 positive leukocytes and also MRP-8/14 deposition on the endothelial surface. This figure is reproduced from (97).
1.2.5.5 Mechanism of protein release

Although many studies have reported finding MRP-8 or MRP-14 extracellularly, alone or in complex, no reports have dealt with the actual mechanism of MRP-8/14 release. Until recently, it was unclear whether a specific release mechanism existed at all, or whether cell necrosis at sites of inflammation was the major source of extracellular MRP protein. Although none of the S100 family has a signal sequence (128) for secretion via the Golgi/ER route, it was thought that a specific release mechanism must exist given the accumulation of protein in monocyte culture without cell death (164).

A protein kinase C activated secretion mechanism was reported in human monocytes (213). MRP-8/14 secretion could also be stimulated via GM-CSF, IL-1β or LPS stimulation, confirming the physiological relevance of the study. Blocking of vesicular trafficking through the Golgi or ER did not prevent secretion, suggesting an alternative secretion pathway. This pathway was tubulin-dependent and MRP-8/14 proteins could be seen to be associated with the tubulin microfilaments.

The Th2 cytokines IL-4, IL-10 and IL-13 affect the release of MRP-8/14 from peripheral monocytes (164). IL-4 and IL-10 given separately, but not IL-13 decreased the amount of MRP-8/14 complex and MRP-14 released from peripheral monocytes, as measured by sandwich ELISA; the effect of IL-4 and IL-10 synergised. When monocytes were cultured for 7 days with GM-CSF, to induce maturation towards a macrophage-like phenotype, they expressed more MRP protein but were less sensitive to IL-4 or IL-10 inhibition and were again insensitive to IL-13 inhibition. This suggested that activated, differentiated cells lose some inhibitory controls.

1.2.5.6 Growth inhibition

The MRP-8/14 complex has been shown to inhibit the growth of various microorganisms including fungi, yeast, bacteria and human B-cell lines (176, 242, 247). In these studies the complex is referred to as calprotectin. Later studies showed that this fungistatic activity on C. albicans occurred at 20μg/ml MRP-8/14, but that no fungicidal activity could be detected up to 100μg/ml. Also, the fungistatic activity was 1μM zinc-inhibitable although this was not considered due to zinc sequestration by MRP-8/14. Dissection of function was not conclusive as the complex and MRP-14 alone were found to be active (190). In contrast, other reports suggests that the candidastatic activity of calprotectin is indeed mediated by zinc chelation (46, 225).
Interestingly, one study has shown that although muMRP-14 can bind zinc, it is not candidastatic (211).

1.2.5.7 Human MRP-14 functions – peripheral analgesia and cell adhesion

The C-terminus of MRP-14 is identical to neutrophil immobilising factor (NIF) (73) and has homology with the contact domain of high molecular weight kininogen (HMWK) (98). HMWK is involved in the initiation of intrinsic coagulation. It was shown that MRP-14 could compete with HMWK and thereby increase coagulation times although the functional significance of these observations are unclear.

MRP-14 has been proposed to have analgesic properties. An investigation of the role of MRP-14 in pain perception using inflammatory pain caused by intraperitoneal administration of 0.6% acetic acid (60mg/kg) and central pain caused by a 50°C hot plate was performed (79). The injection of 0.8μg MRP-14 but not MRP-8 was found to decrease the perception of acetic-acid mediated inflammatory pain, but not heat-mediated central pain. Further, mice injected intraperitoneally with 0.5ml 5% glycogen solution experienced less pain than those mice injected with saline or acetic acid even though all treatments responded equally to central pain. However, the difference in perceived inflammatory pain could be abolished using either an anti-neutrophil or anti-MRP-14, but not anti-IgG antibody. This was given as evidence for a direct role of MRP-14 in inflammatory but not central pain perception.

There were various problems with this study including, a failure to assess the effect of an anti-MRP-8 antibody on nociception and a failure to assess the effect of anti-MRP-14 antibody on the number of cells infiltrating the peritoneum. The anti-neutrophil antibody reduced the infiltrate to control levels. Also, in the mouse, peritonitis is thought to be a most distressing inflammatory stimulus which lowers general vitality and could well account for the decreased perceived nociception (personal observation and Julie Bee, ICRF, personal communication). When the inflammation was relieved after removal of the peritoneal infiltrate with anti-neutrophil antibody, the nociception returned to normal levels. Therefore, this study and its conclusions must be treated with caution.

Although recombinant human MRP-8 and MRP-14 protein was first produced in 1987 (200), the first functional study utilising recombinant human proteins was published only recently. MRP-14 has been shown to be a potent activator of
neutrophil adhesion (198). MRP-14 activates the β2-integrin Mac-1 to bind to fibrinogen, by affinity (strength of interaction) rather than avidity (number of Mac-1 molecules on the cells surface) changes. This stimulated adhesion was blocked by the addition of MRP-8 to the reaction which was found to generate MRP-8/14 complex. MRP-14 induced adhesion was mediated by a pertussis toxin sensitive pathway but not by direct binding to Mac-1. Investigations of neutrophil function demonstrated that MRP-14 did not cause calcium flux, shape change, degranulation, loss of L-selectin, superoxide burst or chemotaxis. This work strongly suggested that the MRP-8/14 complex is not biologically active in vivo and may actually represent a natural "brake" on the pro-adhesive properties of MRP-14. As the majority of extracellular MRP-8 and MRP-14, in vivo, is found as the complex form, it is of interest that deletion of the first 4 amino acids of human MRP-14, the natural truncated form, was found to decrease the strength of the MRP-8/14 interaction in a yeast two-hybrid system (208). This complex may dissociate more easily and provide a source of monomeric MRP-14, which may then act as an integrin activating agent and thereby increase cell recruitment to an inflammatory foci.

1.2.5.8 Murine MRP-8 and MRP-14

In contrast to the human proteins, less is known about the prevalence of a cellular MRP-8/-14 complex in mice. Expression of MRP-8 and MRP-14 do overlap (140) and it has been reported that MRP-8 and MRP-14 complex does exist as a 1:1 heterodimer in bone marrow cells (80). Interactions between MRP-8 and MRP-14 in a yeast two hybrid system have also been reported (208). Interestingly, a recent report suggests that a MRP-8/14 complex is found in bacterial abscesses, but that it is mainly disulphide-linked with a small proportion that is calcium-dependent (129). This contrasts with the human situation in which the majority of complex is linked non-covalently (67, 255). Although the cellular source of the complex was not identified, the likely candidates were neutrophils as they were the major infiltrating cell type. This evidence was strong as it used a monospecific MRP-14 antibody to immunopurify MRP-14 and MRP-8 from an abscess protein solution. The 24kDa, presumably MRP-8/-14 complex was thought to be formed primarily through covalent bonds and only secondarily through non-covalent interactions. Approximately 71% and 64% of the
total amounts of MRP-8 and MRP-14, respectively, were thought to be in complex form.

A later report also suggested that MRP-8 and MRP-14 could form a complex (127). MRP-8 and MRP-14 were synthesised as His-fused recombinant proteins and boiled together for 5 minutes in 50mM CaCl₂ / 5% β-mercaptoethanol and dialysed into various 50mM calcium containing buffers. Subsequent SDS analysis showed the formation of approximately 15% (of total protein) MRP-8/14 heterodimer. The heterodimer, but not MRP-8 or MRP-14 alone, in the presence of calcium (but not EDTA), was then shown to bind arachidonic but not oleic acid. The authors suggested a role for MRP-8/14 as either a transporter or sequestrator of inflammatory mediators. A similar phenomenon has been reported in human keratinocytes (237).

1.2.5.10 Function of murine MRP-8 (CP-10)

MRP-8 (also known as CP-10) was purified (137), and cloned (140) at exactly the same time by two groups. The protein was isolated from concanavalin-A activated spleen cells and the cDNA cloned from a spleen phage library. This protein was found to have extremely potent chemotactic activity (0.1fM), for neutrophils in vitro (137). In an in vivo footpad model, MRP-8 recruited primarily neutrophils between 4 to 8 hours but also mononuclear cells after 24 hours (138). Structure-function studies revealed that the variable hinge region was chemotactic and also demonstrated the functional dissimilarity between human and mouse MRP-8 as the human MRP-8 hinge region was inactive in chemotaxis assays (138).

MRP-8 chemotaxis is unusual in that it does not cause activation of neutrophils i.e. superoxide production or granule release (56). Therefore, MRP-8 is considered to be a pure chemoattractant, like TGF-β1 (50). Pure chemoattractants do not cause calcium fluxing or cell activation in contrast to classical chemoattractants such as fMLP and IL-8 which activate cells via increased intracellular calcium levels and cause superoxide release (90).

MRP-8 can also be found after inflammation has been induced with other agents. For example, 3 hours after a 3ng injection of LPS into a mouse footpad, MRP-8 was found in discrete areas on the surface of endothelial cells, that also co-expressed factor VIII (56). The distribution of MRP-14 was not analysed. The MRP-8-endothelial association closely parallels the situation found in humans where MRP-8/14 complex
coats the endothelial surface, in association with trafficking leukocytes (103) (see Figure 1.2B, page 41).

MRP-8 may also have pathogenic roles. MRP-8 recruited monocytes and macrophages expressed increased scavenger receptors and cholesteryl esters in response to acetylated LDL (147). This may suggest a pathogenic role for MRP-8 in the foam cell generation associated with atherosclerosis. MRP-8 also causes intracellular increases in F-actin within neutrophils, resulting in size increase and shape change with pseudopodia within 90 minutes (50). It was proposed that this may contribute to capillary cell retention and hence inflammation initiation within the vasculature (147).

1.2.5.11 Transcriptional control of MRP-8 and MRP-14

LPS affects the transcription of MRP-8 but not MRP-14 in myeloid cell lines and elicited macrophages but not bone marrow derived macrophages (107). Sodium periodate elicited macrophages, when stimulated with 10ng/ml LPS, upregulated MRP-8 but not MRP-14 mRNA and secreted MRP-8 protein. When these cells were stimulated a second time, both MRP-8 mRNA and protein levels markedly decreased. Resting monocyctic cell lines such as RAW 264.7 and WEHI 265.1 only expressed MRP-8 mRNA and protein after LPS priming (0.1ng/ml) and did not express MRP-14 mRNA or protein at any time. The mRNA up-regulation was not mediated by increased half-life, suggesting transcriptional regulation. This LPS regulation of MRP-8 gene and protein expression was proposed to be a physiologically important mechanism in host defence.

A later study with murine endothelioma cell lines showed similar phenomena (283). In a brain endothelioma line, bEND-3, MRP-8 mRNA was upregulated at 24 hours by 100ng/ml LPS, 150U/ml IL-1α and 340U/ml IL-1β but not 12U/ml TNFα whereas MRP-14 mRNA was upregulated at 24 hours LPS, IL-1β and TNFα. Synthesis of MRP-8 protein was via de novo protein synthesis but, in contrast, no MRP-14 protein could be detected after any stimulus. Also, 17% of microvessels in DTH-footpads were found to cytoplasmically express MRP-8. This study complements the macrophage study (107) and suggests that MRP-8 may be an important cytokine in LPS-mediated leukocyte recruitment.
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1.2.5.12 Murine MRP-14

MRP-14 was cloned in 1992 (140) although native protein was not purified until 1996 (211). Various studies with recombinant MRP-14 have since been published (1, 79, 127, 209, 281). Native MRP-14 has been shown to undergo post-translational modifications including N-terminal methionine removal, N-terminal acetylation, internal disulphide bonding at cys^{79} and cys^{90} and 1-methylation of his^{106} (211). This may be important for recombinant MRP-14 function. Interestingly, both human and mouse MRP-14, to a greater extent than human or mouse MRP-8 have been shown to bind zinc (211).

Some investigations into cell growth inhibition have also been performed using the mouse MRP proteins. In contrast to the human data, solutions of MRP-8 and MRP-14 (10μg/ml each and 40μg/20μg per ml, respectively) failed to inhibit the growth of either *E. coli* or *C. albicans* (129). Murine calprotectin has yet to be assessed. Indeed, the major group analysing rat calprotectin function has not yet published results with murine protein (285).

1.2.5.13 Pro- and anti-inflammatory properties of murine MRP-14

Anti inflammatory properties have been demonstrated for MRP-14. Granulomas are persistent inflammations, characterised by a class of differentiated macrophages, called epithelioid cells (125) and a mononuclear infiltrate (170). MDF (macrophage deactivating factor), is an 11Kda factor which inhibits O^{2-} release by activated mouse peritoneal macrophages and human neutrophils (40), which can be expressed in epithelioid cells (171). Epithelioid cells, formed after 14 days, were found to express MRP-14 but not MRP-8 (1). MDF activity, as assessed by decrease in oxygen free radical release, was stimulated by 100ng/ml human or mouse recombinant MRP-14 but not 100ng/ml human or mouse recombinant MRP-8. This MDF-like activity was also inhibited by an anti-MRP-14 but not an anti-MRP-8 antibody (1). Whether MRP-14 is MDF was not established.

In contrast, another study has suggested a pro-inflammatory role for murine MRP-8 and MRP-14 in granuloma cell recruitment (281). MRP-8 and MRP-14 were identified as major components of GIF (granuloma initiation factor), which can be purified from murine schistosomiasis liver granulomas. GIF can then be used to transfer granulomas to other mice (112). Recombinant MRP-8 and MRP-14, and MRP-8/14
complex were generated and coupled to Affi-Gel beads. The beads were then implanted subcutaneously and a neutrophil influx induced at times up to 4 days with MRP-8, MRP-14, MRP-8/14 and GIF; by day 40-50 a granuloma type reaction with large macrophages had developed, but only with GIF. Inoculation with the MRP-8/14 complex caused a cellular infiltrate which was larger than either MRP-8 or MRP-14, which were comparable. Also, inoculation with the complex prolonged the acute neutrophil reaction and caused chronic eosinophilia. It was proposed that MRP-8 and MRP-14 contribute not to granuloma formation, but to cellular recruitment within the granuloma. This was an artefactual study, given that the stimuli (the recombinant proteins) could not be removed from the inflammatory site as they were coupled to beads. Therefore they were constantly recruiting cells. It is probable that the chronic effect of eosinophil recruitment is not physiologically significant. The effect of MRP-14 on granulomas seems unclear.

1.2.5.14 Rat MRP-8 and MRP-14

Rat MRP-8 and MRP-14 have also been identified, cloned and expressed (115). Rat MRP-8 and MRP-14 were found to be 89 and 113 amino acids in length, respectively. Rat MRP-8 is 81% homologous to mouse and 63% homologous to human MRP-8 whereas rat MRP-14 is 75% homologous to mouse and 60% homologous to human MRP-14. This suggests that the rat proteins will bear more functional similarity to the mouse proteins, rather than the human proteins.

As has been found with human calprotectin, rat calprotectin has also been found to show growth inhibitory effects on bone marrow cells and macrophages and growth inhibitory and cytotoxic effects to the MM46 mammary tumour cell line at concentrations of 100μg/ml. These effects were completely abrogated with 2μM zinc (287). Later studies have suggested that 100-200μg/ml calprotectin can induce apoptosis in the mouse EL-4 lymphoma and human MOLT-4 leukaemia cell lines; this effect was abrogated with 10μM zinc (286). Similar findings were found with murine embryonic and human dermal fibroblasts (285).

In summary, evidence suggests that MRP-8 and MRP-14 are involved in cell migration and at least in the mouse, cell recruitment. Evidence about other functions including
inhibition of cell growth and analgesia is less clear. Other unknown functions may exist, especially as these proteins are so highly expressed in myeloid cells.

1.2.5.15 MRP-6

Another member of the MRP family was identified in 1994 as an 8% component of pig granulocytes (55). This protein, originally called calgranulin C was especially interesting because of its zinc binding characteristics. Upon zinc binding, calgranulin C bound two calcium ions with a much higher affinity ($K_a$ values of approximately $3 \times 10^7$ and $6 \times 10^4 / M$) than in the absence of zinc where the protein bound one molecule of calcium. ($2 \times 10^4 / M$). Moreover, this was associated with a change in the shape of the molecule, suggesting that calgranulin C might be involved in calcium-dependent signalling events.

The human homologue of pig calgranulin C, named $p_6$ (MRP-6), was identified in 1995 from neutrophils and monocytes using a rabbit polyclonal antibody raised against human MRP-8 (86). $p_6$ was named, as MRP-8 and MRP-14 were, by its apparent SDS-PAGE molecular mass. MRP-6 constituted 5% of the cytosolic protein. Two isoforms were isolated, the major form ($p_6b$) with a $pI$ of 6.3 and the minor form ($p_6a$) with a $pI$ of 6.2. Only $p_6b$ was found to bind calcium. Both isoforms of $p_6$ could be induced to translocate to the membrane, with MRP-8 and MRP-14 after exposure to a calcium flux stimulator, such as fMLP. This membrane association was found to be dependent on calcium and the cytoskeleton.

MRP-6 was completely sequenced and given the S100A designation, S100A12 in 1996 (114). The 92 amino acid MRP-6 does not form higher order complexes with itself and is the most divergent S100 family member yet identified, with only 46% amino acid identity with its closest relative, MRP-14. Identity with MRP-8 is only 40% (173).

The gene and cDNA clones for human MRP-6 were characterised and sequenced in 1996 (277). The MRP-6 gene was typical of the S100A family with a three exon and two intron structure, localised to chromosome 1q21.2-22. The cDNA sequence was 166bp in total with an open reading frame of 276bp.

MRP-6 has been shown to co-localise with MRP-8 and MRP-14 and neutrophil defensin proteins, on the surface of the tissue-dwelling *Onchocerca volvulus* parasite (173). *Onchocerca volvulus* is the causative agent in onchocerciasis, or river blindness. This suggests a role for the MRP proteins in host-parasite interactions.
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The bovine homologue of human MRP-6 and pig calgranulin C, called CAAF1, was identified in 1996, from bovine amniotic fluid (101). It was expressed in neutrophils, some macrophages and also in squamous epithelial cells (such as the oesophagus, amnion and skin). The authors suggested that CAAF1 may be being secreted into the amniotic fluid.

Also in 1996, rabbit MRP-6 was isolated and cloned (282). This study discovered rabbit MRP-6 whilst investigating a role for neutrophil sulfite production, which is thought to be important as an anti-bacterial agent. Inorganic $^{35}$S-sulphate was added to neutrophils. The neutrophils reduced the sulphate to sulphite and some proteins became labelled with $^{35}$S. These labelled proteins were then identified by anion exchange chromatography. 83% of the $^{35}$S was found to be incorporated as part of MRP-6, suggesting a role for MRP-6 in sulphate reduction. The MRP-6 with the most label was found extracellularly, suggesting a role for sulphate reduction in secretion.

1.2.5.16 MRP-6 is EN-RAGE, a potent pro-inflammatory molecule

As has been the case for MRP-8 and MRP-14, almost no functional data is available for MRP-6, from any species. Very recently, a seminal paper has been published which sheds new light on the whole S100 field. Bovine MRP-6 was found to specifically bind RAGE, leading to a proposed name for MRP-6 of EN-RAGE (extracellular newly identified RAGE binding protein) (102).

RAGE (receptors for advanced glycation end products) is a promiscuous member of the immunoglobulin superfamily which binds AGE (advanced glycation end products) (196, 232). AGE are formed by glycoxidation reactions which form protein adducts. RAGE was identified due to its AGE binding ability and was highly expressed on cells found in conditions such as diabetes (29) and renal failure (183) where AGE are found. Lactoferrin (231) and Aβ (amyloid-β peptide), the cleavage product of the β-amyloid precursor protein also bind RAGE (278). Aβ is associated with the pathogenesis of Alzheimer’s disease (236). RAGE ligation by AGE is associated with p21$^{ras}$ activation, followed by activation of MEK and MAP kinases and recruitment of NF-κB (141). This suggests that AGE and Aβ can mediate cellular activation via RAGE. AGE can also elicit disposal rather than biological activation responses. The scavenger receptor, found on macrophages, mediates AGE uptake and degradation, so preventing AGE ligation of RAGE (134).
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The isolation of EN-RAGE was pursued after consideration of RAGE tissue expression patterns. RAGE is highly expressed during CNS development (106) but it is also highly expressed in the adult lung. This led to the screening of lung for RAGE ligands and the identification of EN-RAGE (MRP-6) (102).

EN-RAGE was found to be upregulated 4-fold in PBMCs and 2.5-fold in Jurkat cells after CD3/CD28 crosslinking. EN-RAGE itself could also activate PBMCs to release the T lymphocyte survival factor, IL-2. This result was particularly interesting as none of the MRP proteins have previously been found in lymphocytes.

EN-RAGE was also shown to activate endothelial cells, by increasing 4-fold VCAM-1 and 2-fold ICAM-1 expression levels on HUVECs at 8 hours, which could then mediate increased adhesion of Molt-4 cells. This endothelial cell activation was associated with an intracellular increase in NF-κB.

EN-RAGE was also shown to activate monocytes, in terms of inducing maximal chemotaxis at 95nM. Further, analysis using the BV-2 macrophage cell line demonstrated that EN-RAGE could cause the release of TNF-α and IL-1β, which was associated with nuclear translocation of NF-κB. Direct binding of EN-RAGE was shown by radioligand displacement assays using a truncated soluble RAGE or anti-EN-RAGE antibody. Interestingly S100B was also shown to bind to RAGE.

In vivo investigations showed that 30μg of intravenous EN-RAGE could induce the upregulation of VCAM-1 at 12 hours in the lung whereas 10μg of EN-RAGE caused a cellular influx 24 hours after injection in the mouse footpad model. Further investigations of the role of EN-RAGE/RAGE interactions in a methylated BSA (non-RAGE ligand) model of delayed type hypersensitivity showed that the hypersensitivity reaction could be blocked by soluble RAGE and anti-EN-RAGE or anti-RAGE antibodies. Further evidence for the role of RAGE in inflammation was shown using the IL-10 KO colitis model in which IL-10 KO mice were injected intra-peritoneally with either methylated BSA (mBSA) or soluble murine RAGE (sRAGE) every day for 6 weeks. 80% of mice treated with mBSA had strong inflammation in the colon whereas 80% of the sRAGE treated mice had no inflammation. These data suggested that RAGE receptors and EN-RAGE play a pivotal role in the cellular activation of inflammation.

This paper is particularly exciting as it suggests that RAGE can be activated by various members of the S100 family, such as S100B. Interestingly, specific cell surface binding of MRP-8 and MRP-14 has recently been demonstrated with monocytic, T and
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B cell leukaemia cell lines (130). As EN-RAGE stimulates monocytes and lymphocytes, the reported MRP-8/14 binding may actually represent binding of MRP proteins to the receptor molecule, RAGE.

Future work may link the elevated serum levels of MRP-8 and MRP-14 with inflammation, mediated by RAGE. Indeed, the function of the MRP-8/14 complex, the predominant MRP form found in the serum may finally be elucidated.

Before significant MRP-8 and MRP-14 functional data was accumulated, it was proposed that they might act as “calcium sinks” (93) due to their high expression levels (3mM in neutrophils (67)). This would protect cells from the deleterious effects of prolonged intracellular calcium elevation. Hopefully, the preceding section has shown that this is unlikely to be the true function of the MRP proteins.

The aims of this thesis are to further investigate the role of MRP-8 and MRP-14 in vitro and in vivo. One of the ways this has been attempted has been via gene targeting. The next section aims to introduce the history, current status and future of gene targeting.

1.3 Gene targeting

1.3.1 A brief history of gene targeting

The first step taken in the development of null-expression, or “knockout” animals was taken in 1968 with the generation of the first chimeric mouse (77). This meant that a live animal could be produced from 2 separate sources of cells, which led to the idea of altering one set of cells in order to propagate an introduced mutation. However, to do this, a chimera must be able to transmit the introduced mutation.

Embryonic stem cells were derived from the inner cell mass of day 3.5dpc blastocysts and shown to be pluripotent cells that could repopulate blastocysts (71, 174). The first chimera capable of transmitting genes from re-introduced cells was created in 1984 from ES cells (26). Once the technology had developed thus far, with the cells in which mutations could be generated, ES cells, and from those cells, the ability to create chimeras and transmit mutations into later generations, the missing link was how to mutate genes of interest.

The method developed to introduce mutations of interest into the genome was gene targeting. This is based on the phenomena of homologous recombination in which
an exogenous piece of DNA can recombine with and replace an endogenous segment of DNA. It is possible to introduce into the homologous targeting DNA, a mutation of interest, which can then be introduced into the genome. Gene targeting was first demonstrated in mammalian cells by adding a missing 3' - portion of the thymidine kinase gene as part of an exogenous vector, to an endogenous 5' - portion in L-cells (fibroblast like cell line) (156). Another pioneering group demonstrated a targeting event at the β-globin locus in a β-globin expressing erythroleukaemia cells and a β-globin non-expressing bladder carcinoma line (241). This demonstrated the important point that genes did not need to be expressed to be targeted. Later experiments showed that ES cells could be targeted with functional results, in this case correction of a mutant HPRT gene (62). Finally, it was demonstrated that ES cells could be targeted and then re-introduced into blastocysts, produce chimeras and then transmit the mutation to the next generation (257). This was particularly important as it demonstrated that ES cells could be cultured and then targeted without losing the all-important ability to transmit the mutation through chimeras to the next generation.

1.3.2 Future of knockout technology

Gene targeting is now a standard method in laboratories throughout the world. The majority of knockouts use either a replacement (true replacement or deletion events) or an insertion vector. The main difference between a replacement and insertion vector is that replacement vectors are linearised outside, and insertion vectors are linearised inside, the region of homology, prior to electroporation or transfection. This means that in a replacement vector, vector sequences are lost and the homologous sequences replace the endogenous sequence. In an insertion vector, the whole construct, including vector is targeted to and included in the target locus, leading to exon duplication. These strategies are adequate to answer loss of gene function questions. However, to evaluate the function of gene necessary for development or to evaluate the effect of subtle gene mutations, loss of function mutations are relatively useless and other techniques have been developed to assess these questions.

Two techniques based on existing targeting technology will be discussed. These are the “hit and run” and “double targeting” techniques. The “hit and run” technique was originally reported in 1991 (92) to target the Hox genes. The technique was useful as it targets the gene of interest but causes a nucleotide change and not abrogation of
function. Moreover, the technique results in removal of the resistance cassettes so that
the influence of strong viral promoters or enhancers, commonly found in resistance
cassettes are removed from the milieu, ensuring that the transcription of neighbouring
genes is not altered. This technique uses an insertion vector with both positive selection
(neomycin resistance cassette) and negative selection (thymidine kinase, which makes
cells FIAU sensitive). The selection cassettes are sited outside the region of homology.
Both cassettes and the mutant homologous sequence are targeted to the homologous
locus and become incorporated into the genome. This event can be selected for by
neomycin resistance. Then a intrachromosomal recombination event occurs between the
targeted and non-targeted chromosome resulting in the loss of the two cassettes and the
vector sequence. This can be selected for with FIAU resistance and neomycin
sensitivity and screened for by Southern hybridisation. In this way, subtle changes can
be introduced into loci.

The “double targeting” technique is similar to the “hit and run” technique but
does not rely on intrachromosomal recombination (215). Instead the technique utilises a
HPRT deficient ES-cell line. The first targeting vector contains homologous regions to
the exon of interest and its flanking regions and a TK cassette, outside the region of
homology. The exon itself is replaced with a HPRT minigene. If the constructs targets
to a site in the genome other than the site of interest, the TK cassette is not lost. This
results in TK expression and FIAU sensitivity. Targeting results in an ES cell where the
exon of interest is replaced with a HPRT minigene, allowing positive selection in HAT
(hypoxanthine, aminopterin, thymidine) and negative selection with FIAU. The
correctly targeted clone can then be re-targeted with a second construct in which the
HPRT minigene is replaced with the subtle mutation of interest. Positive recombinants
can then be identified by HAT sensitivity. As with the “hit and run” method, the
“double targeting” method can hence be used to introduce subtle mutations into genes.

Standard removal of a functional gene using replacement or insertion vectors are
of no use in the evaluation of the function of a gene necessary for development. Also
“Hit and run” and “Double targeting” have limited use if the gene of interest requires full
function during development. However, the development of the Cre-loxP strategy has
provided a new method to evaluate the function of such developmentally important
genes.
The Cre-loxP recombination system in bacteriophage P1 was adapted to target genes in mammalian cells (227) and was first used to create knockout mice in 1993 (85). When used to flank a sequence, loxP sites can be used to remove (if the loxP sites are both facing 5’- to 3’-), or invert the sequence (if one loxP site points 5’- to 3’- and the other 3’- to 5’-) after the addition of Cre recombinase. The key point is at what point and by what method the investigator chooses to add the Cre recombinase.

There are various ways that the manipulation of Cre recombinase expression can affect the gene targeting. Cre can be coupled to a tissue specific or temporally specific promoter. This construct can then be targeted as a transgene into mice and the Cre transgenic mice crossed with the floxed mice (mice carrying two copies of the loxP targeted gene of interest). In this way, a lethal gene could be allowed to be present and expressed during development but then be removed by the expression of Cre at a certain time throughout the body, or only in certain tissues, dependent on the promoter used. A recent example of this is the knockout of the glutamate receptor subtype, NMDAR1, in only the CA1 pyramidal cells of the hippocampus (260, 261). This study used Cre recombinase fused to the αCaMKII promoter as this gene is known to be expressed post-natally in the forebrain (35). The conclusion from the original NMDAR1 knockout which used conventional loss of function targeting, was that NMDAR1 was required for development (155). The temporal and tissue specific expression of Cre recombinase allowed the role of NMDAR1 in the forebrain, rather than in the whole body and also post-natally, rather than in development, to be studied. Other tissue coupled promoters are available and it is likely that more will become available with time.

The other methods of expressing Cre recombinase at times and in places chosen by the investigator are to use constructs where Cre expression is coupled to either tetracycline repression (96) or tamoxifen induction (182), (288).

In the tetracycline case, the Cre cassette consists of a TetR/VP16 hybrid gene coupled to Cre (251). Tetracycline is given to the mice prophylactically, which represses Cre expression. At the point in time that Cre expression is required, the tetracycline is removed from the drinking water, the Cre expression is derepressed and the floxed gene is removed.

In the tamoxifen case, the Cre cassette consists of Cre recombinase linked to a mutated ligand-binding domain of the murine oestrogen receptor. The oestrogen
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The receptor does not bind the biologically significant 17 beta-oestradiol but does bind tamoxifen (288). Upon tamoxifen binding, Cre expression is induced.

Both systems have the potential for further refinement, such as tissue targeting. Indeed, a tamoxifen-inducible Cre has been localised to the epidermis and some other stratified squamous epithelia using the human keratin 14 (K14) promoter (263). This then allows the topical application of tamoxifen to trigger gene removal and hence removes the systemic effect of tamoxifen from the experimental system. Also, a tetracycline inducible system has been localised to the epithelial cells of secretory organs and skin, such as seminal vesicle, salivary gland, and Leydig cells. This was achieved using the human cytomegalovirus immediate early 1 (HCMV-IE1) gene promoter (96).

This section has described the development, current implementation and future of gene targeting. The next section will briefly describe the impact and some of the findings of gene targeting in the field of adhesion and leukocyte biology.

1.3.3 Knockouts in adhesion

The use of conventional knockout models has generally produced either confirmation of the function of an adhesion molecule or it has merely shown that a molecule is necessary for development. However, some surprising findings have been made with knockout mice and the use of knockouts has allowed further dissection of the function of molecules. The field of adhesion molecule biology now awaits the application of conditional knockouts to finally dissect out the functions of integrins that have been shown to be involved in development, such as $\alpha_4\beta_1$ (279) and $\alpha_5\beta_1$ (280). This section will be restricted to the description of gene ablation experiments that are directly relevant to neutrophil adhesion and migration.

LFA-1 (CD11a) has been functionally deleted by several groups. Initial reports focused on the immune functions of LFA-1 (233), showing that LFA-1 KO mice had decreased proliferation after mitogen stimulation, in mixed lymphocyte reactions and failed to reject immunogenic tumours. However, cytotoxic responses towards viruses were normal. LFA-1 KO leukocytes were less able than heterozygous leukocytes to emigrate into the peritoneum at both 4 hours and 24 hours after 1.5ml 4% thioglycollate insult. Also, LFA-1 deficient mice did not undergo delayed-type hypersensitivity responses (DTH), measured by ear thickness changes after challenge with DNFB after pre-immunisation on the abdomen.
A recent report confirms the involvement of LFA-1 in lymph node localisation, showing that LFA-1 deficient lymphocytes are compromised in their lymph node homing abilities with LFA-1 KO peripheral lymph nodes (pLN) having 30% of the number of cells found in LFA-1 WT pLN (19). KO cells were found to have impaired trafficking, compared to WT cells. The compensatory mechanisms allowing lymphocyte homing to lymph nodes were assessed. It was found that in the KO, $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins binding to VCAM-1 compensate for LFA-1 in peripheral lymph node migration although VCAM-1 expression was the same in both the WT and KO mice.

Various studies have also been performed with Mac-1 (CD11b) deficient mice. One study (51) found a decreased chemoattractant-induced binding to endothelium, as might be expected. However, there was a greatly increased thioglycollate-induced neutrophil recruitment to the peritoneum. These cells had a decreased rate of apoptosis versus WT cells. A role for Mac-1 in apoptosis was confirmed by the induction of apoptosis in normal neutrophils with opsonised particles and the inhibition of this effect with anti-Mac-1 antibodies.

In contrast, another study found no increased peritoneal recruitment of leukocytes at up to 4 hours after thioglycollate challenge for KO versus WT Mac-1 mice (163). However, the accumulation of cells at 4 hours in response to thioglycollate could be inhibited 78% in the KO and 58% in the WT with the anti-LFA-1 antibody KBA; treatment of WT mice with the Mac-1 antibody M1/70 decreased peritoneal infiltration by 30%; treatment with KBA and M1/70 did not increase the inhibition seen with KBA alone. Taken together, these data demonstrated that LFA-1 is the most important integrin in peritoneal emigration. Also, phagocytosis of opsonised zymosan particles was decreased in KO versus WT animals and adhesion to Mac-1 ligands such as fibrinogen and haemocyanin was abolished. Furthermore, degranulation was decreased in KO versus WT mice. No significant differences in peripheral leukocyte populations, in terms of numbers or percentages were noted.

The first report of CD18 deficient mice described a hypomorphic, rather than knockout mutation in which KO mice expressed 2% or 16% of WT levels of CD-18 protein, on resting and stimulated leukocytes, respectively (272). The basis of this failure to generate a complete knockout was based on an insertion rather than replacement event in which gene splicing allowed the formation of some functional
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CD18 mRNA using a cryptic bacterial promoter present in the inserted vector. CD18 KO mice had increased blood neutrophils but these cells were less able than WT cells to emigrate to the peritoneum, as measured at 4h after 1ml thioglycollate challenge.

A later study examined the phenotype of completely CD18-deficient mice (184). CD18 KO mice had 11 times more neutrophils in the blood, and almost 4 times the total leukocytes than did CD18 WT control mice. As was expected from the paradigm of CD18 mediated leukocyte adhesion and migration, no infiltration was observed in croton-oil induced dermatitis in the CD18 KO animals versus the large infiltration found in the CD18 WT animals. The surprising finding in this study was that neutrophil migration into the lungs, in response to *S.pneumonia* pneumonia was not different between CD18 KO or CD18 WT animals. Moreover, there was no significant difference in the neutrophil recruitment to the peritoneum after *S.pneumonia* or thioglycollate-insult. These results did not mimic the phenotype of the human LAD patients, suggesting that animals that have developed without CD18 have also developed an adaptive mechanism to allow leukocyte migration and the resolution of infection into the lungs or peritoneum, but not the skin. However, this study must be interpreted in the light of the 11-fold increase in circulating neutrophils.

When CD18-129/Sv knockout mice were bred onto a PL/J background, a psoriatic-like condition developed; this condition was absent in the original CD18-129/Sv mice (33). This suggests that modifier genes that interact with integrins exist in the Sv but not PL/J mice that modulate the pathology.

The study of CD18 deficient mice was particularly interesting as it mimics the human condition of leukocyte adhesion deficiency (LAD). LAD patients have a severe phenotype with impaired wound healing, lack of pus formation and recurrent infection. The molecular basis of the disease is a deficiency in CD18, either an absence (7) or presence accompanied by lack of function (104).

ICAM-1 deficient mice were found to have leukocytosis, impaired neutrophil emigration in chemical peritonitis and decreased contact hypersensitivity induced by 2,4-dinitrofluorobenzene. Immunologically, ICAM-1 KO mice had mild granulocytosis and were unable to stimulate the mixed lymphocyte reaction, although they proliferated normally as responder cells (238). Later studies showed that absence of ICAM-1 decreased the extent of reperfusion following ischaemia-induced injury in the CNS (48) and kidney (121).
Using knockout models, a role for ICAM-1 has been uncovered in septic shock (276). ICAM-1 KO mice were found to be resistant to LPS-induced septic shock, in contrast to ICAM-1 WT mice, although neutrophil accumulation was detected in the liver. Normal cytokine profiles were found in both the ICAM-1 WT and KO mice suggesting that a defect in leukocyte adhesion was responsible for the KO insensitivity to LPS. This was probably a failure in transendothelial migration.

Another molecule involved in the transmigration of neutrophils, across both endothelium and epithelium is CD47 (IAP). The functional relevance of CD47 has been confirmed with gene targeting. CD47 KO mice had a decreased response, in terms of cell recruitment and decreased bacterial killing, to peritoneal *E. coli* infection as compared to CD47 WT mice (157).

The selectin molecules have also been deleted. L-selectin-KO mice were found to exhibit the expected defects in lymphocyte rolling and hence decreased adhesion to high endothelial venules. L-selectin KO lymphocytes also had decreased homing to various peripheral lymphoid organs including mucosal lymph nodes, Peyers patches and spleen. Examination of neutrophil function with the thioglycollate-elicited peritonitis model demonstrated that peritoneal neutrophil accumulation was compromised in the L-selectin KO versus WT animals, at all early timepoints measured from 1-4 hours (10).

P-selectin KO mice also have defects in leukocyte rolling, increased circulating leukocyte levels and decreased neutrophil recruitment to the peritoneum after thioglycollate insult, as compared to P-selectin WT animals (175). Analysis of the peritoneal emigration defect found the effect to be most pronounced at 1 and 2 hour time points, but only a 2-fold defect at 4 hours. That this peritoneal defect was due to the absence of P-selectin was confirmed by the presence of L-selectin and the inducible presence of E-selectin in the P-selectin KO mice.

E-selectin KO mice were found to have no significant defects in inflammatory models, unless P-selectin antibodies were also used (136). This suggested an overlapping and functionally redundant role for E- and P-selectin. This was supported by the data from E-selectin WT mice treated with an anti P-selectin antibody. These mice had defects in thioglycollate-induced infiltration into the peritoneum at early (2h) but not later (6h) timepoints. This data also suggested that the molecule allowing neutrophil infiltration at 4 hours in the P-selectin KO (175) was indeed E-selectin.
A later study confirmed that E-selectin was functionally redundant in the mouse (153). Using L-selectin KO and P-selectin KO mice, with anti-L-, anti-P- and anti E-selectin antibodies the contribution of each selectin to rolling was elucidated. P-selectin was thought to be important for the initial rolling of leukocytes after injury with the later events mediated by L-selectin. If TNF-α was used to stimulate the endothelium, which has been shown to up-regulate surface expression of E-selectin (21), then rolling could be inhibited by anti-L- and not anti-P- selectin antibodies. This work suggested that there was no functional role for murine E-selectin in rolling, as was also suggested from the knockout work (136).

This section has aimed to demonstrate some of the clarifications and advances that the application of knockout technology has brought to the field of adhesion biology. Although the advances have been great, the advent of the use of conditional knockouts will remove developmental considerations from the knockout phenotype and will further clarify the role of important adhesion molecules.

1.3.4 Project justification and knockout considerations
The initial aims of this project were to characterise the function of MRP-8 and MRP-14 in myeloid cells, particularly neutrophils and also in the immune response, to challenges which elicit myeloid cells. As described in the Introduction, some functions have been ascribed to murine MRP-8 but functional data for murine MRP-14 is sparse. Therefore, it was decided to investigate the function of MRP-8 and MRP-14 by gene inactivation and the production and characterisation of recombinant protein.
Chapter 2: Materials and Methods

2. Materials and Methods

2.1 General Materials

The following materials were standard laboratory chemicals and were all sourced from Sigma (UK).

- SDS
- Sodium hydroxide
- Sodium Chloride
- Water saturated butanol
- HEPES
- Sodium Acetate
- 2-mercaptoethanol
- Ethanol
- Methanol
- Chloroform
- EDTA

2.2 PCR

2.2.1 Materials

- Perkin Elmer 9600 PCR machine
- ICRF recombinant TAQ polymerase (ICRF, UK)
- 1 x PCR buffer 1ml 10x PCR buffer (Promega), 600μl 25mM MgCl₂ (Final concentration 1.5mM) (Promega), 80μl dNTPs (20μl of each 100mM dNTP) (Pharmacia), 8.32ml H₂O.
- Primers (ICRF, UK)
  - MUMRP8-F1 \( T_m \ 61.4°C \) CUACUACUACUACCTTGAGCAACCTCATTG ATG
  - MUMRP8-F2 \( T_m \ 61.4°C \) CUACUACUACUAAATGGTCACCTACTGAGTG TCC
  - MUMRP14-F1 \( T_m \ 61.6°C \) CUACUACUACUAGAGAAGGAGAAATGAA GCCCTG
  - MUMRP14-F2 \( T_m \ 61.6°C \) CUACUACUACUAAAGGAGAAGGAAATGAA GCCCTC
  - MUMRP8-B1 \( T_m \ 60.0°C \) CAUCAUAUCAUGAACCTCCTCGAGTTAAT TGC
  - MUMRP8-B2 \( T_m \ 62.8°C \) CAUCAUAUCAUGTGTCGTCCTTGTTCAGAGA TGC
Chapter 2: Materials and Methods

MUMRP14-B2  T_m 64.2°C  CAUCAUCAUCAUTGGTGGGCAGCAGTCAC  ATG

N.B. All primers used, in all applications throughout this study were purified before first use by ethanol precipitation; this removed small oligonucleotides sequences that may have misprimed and affected the end application.

2.2.2 Method

2.2.2.1 PCR

In general, polymerase chain reaction was performed as described in standard methods (224). Briefly, template DNA was incubated with 170 nanograms of primer and the reactions was performed at 3 degrees below the lowest primer melting temperature for approximately 30 cycles.

The MRP-8 genomic probe used to probe the λ-phage library was generated using the primer pairs 8F1 and 8B2. The MRP-14 genomic probe used in the same application was generated from the primer pairs 14F2 and 14B2. Cycling profiles for both reactions were identical and are detailed below.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>8F1/8B2 or 14F2/14B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’ @ 94°C</td>
</tr>
<tr>
<td></td>
<td>20” @ 94°C</td>
</tr>
<tr>
<td>30 cycles</td>
<td>30” @ 55°C</td>
</tr>
<tr>
<td></td>
<td>60” @ 72°C</td>
</tr>
<tr>
<td></td>
<td>5’ @ 72°C</td>
</tr>
</tbody>
</table>

2.2.2.2 Oligonucleotide melting temperature calculations

This formula was used to calculate all oligonucleotide melting temperatures (15).

\[
T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\% (G+C)) - 675/N - 1(\% mismatch) - 0.65(\% formamide)
\]

N = length of oligonucleotide

[Na+] = concentration of sodium ions, assumed to be 0.1
Chapter 2:- Materials and Methods

2.3 Spectrophotometric quantitation of DNA and RNA concentrations

2.3.1 Method

This method is taken from Maniatis (224). Briefly, samples of DNA or RNA to be tested were diluted in water and their absorbance across a variety of wavelengths measured in quartz cuvettes. These values were used to calculate the concentration and purity of the samples according to the criteria listed in the table below.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>DNA and RNA have peak absorbance here.</th>
</tr>
</thead>
<tbody>
<tr>
<td>260nm</td>
<td>$\text{OD}_{260} = 1 = 50 \mu\text{g/ml DNA} = 37 \mu\text{g/ml ssDNA} = 40 \mu\text{g/ml RNA}$</td>
</tr>
<tr>
<td>280nm</td>
<td>Proteins have peak absorbance here</td>
</tr>
<tr>
<td></td>
<td>Pure DNA preparations should have $\text{OD}<em>{260} : \text{OD}</em>{280}$ between 1.8 and 2.0</td>
</tr>
<tr>
<td>325nm</td>
<td>Particulates and dirty cuvettes cause absorbance here</td>
</tr>
<tr>
<td>230nm</td>
<td>Peptide bonds and aromatics absorb here</td>
</tr>
</tbody>
</table>

2.4 Cloning MRP-8 and MRP-14 cDNAs

2.4.1 Materials

1 x PCR buffer As described in 2.2.1

Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm (°C)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8F</td>
<td>64.3</td>
<td>CAUCAUCAUCAUCATATGCCGCTCTGAACCTGAGAAG</td>
</tr>
<tr>
<td>C8B</td>
<td>66.7</td>
<td>CUACUACUACUAGGATCCTACTCCTTTGCTGTCTT</td>
</tr>
<tr>
<td>C14F</td>
<td>64.3</td>
<td>CAUCAUCAUCAUCATATGGCCAACAAAGACACCTTTCT</td>
</tr>
<tr>
<td>C14B</td>
<td>67.9</td>
<td>CUACUACUACUAGGATCCTACTCCACAGGCCCTTT</td>
</tr>
</tbody>
</table>

Templates

Bone marrow library (D.Simmons, Oxford, UK)

MRP-8 cDNA clone 568827, GenBank AA111306 (IMAGE, USA) (172)

MRP-14 cDNA clone, GenBank AA038078 (IMAGE, USA) (172)

Notes :-

1. cDNA clones #568827 and #472993 were found after a blast search at http://www.ncbi.nlm.nih.gov/dBest/index.html
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2. With reference to primer sequences, those bases in bold letters identify restriction sites, those that are underlined represent areas of homology in the cDNA sequence.

2.4.2 Method

MRP-8 and MRP-14 cDNAs were PCR amplified from a bone marrow cDNA library and 2 EST clones (MRP-8 #568827 and MRP-14 #472993) using the primer pairs C8F and C8B for MRP-8 and C14F and C14B for MRP-14. Cycling was identical for both primer pairs and is described below. PCR products were manipulated as described in 3.2 (on page 106).

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>c8F/c8B and c14F/c14B</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' @ 94°C</td>
<td></td>
</tr>
<tr>
<td>90&quot; @ 94°C</td>
<td></td>
</tr>
<tr>
<td>30 cycles</td>
<td>90&quot; @ 50°C</td>
</tr>
<tr>
<td></td>
<td>60&quot; @ 72°C</td>
</tr>
<tr>
<td></td>
<td>5' @ 72°C</td>
</tr>
</tbody>
</table>

2.5 Protein induction

2.5.1 Materials

Sublocalisation buffer (SL) 50mM TRIS-base pH8, 2mM EDTA, 0.01% Triton X-100.

All inductions in all plasmids required:- IPTG (Calbiochem)

Vector specific inductions:-

- pET-3a Chloramphenicol (35µg/ml) and Carbenicillin (50µg/ml)
- pET-28a Chloramphenicol (35µg/ml) and Kanamycin (33µg/ml)

2.5.2 Evaluating protein sublocalisation

5ml bacteria from an overnight culture were seeded into a new flask containing 50ml media. The culture was grown for 45 minutes at 37°C with shaking after which time the temperature was changed to the induction temperature for 15 minutes. Bacteria were subsequently induced with the appropriate concentration of IPTG and 1.5ml samples were taken at various timepoints.

Samples were fractionated as follows. Bacteria were pelleted via centrifugation in a microfuge at 8064g for 30 seconds after which the supernatant was discarded. 150µl
buffer SL was then added and the sample incubated for 15 minutes at 30°C. The sample was then sonicated and the membrane fraction pelleted by centrifugation for 10 minutes at 8064g. The supernatant was taken into a new vessel; this was the cytosolic component. Both the cytosolic and the membrane and inclusion body-containing pellet were loaded onto gels after dissolving in loading buffer (see section 2.7.1, on page 70).

2.5.3 Induction Method
Bacteria were streaked out from -70°C frozen stocks onto LB-Agar plates, containing the appropriate antibiotics (see section 2.5.1). Single colonies were picked into 50ml medium (plus the appropriate antibiotics) and grown overnight with shaking at 37°C. 8ml of overnight culture was then seeded into 500ml new medium plus antibiotics and the bacteria were grown for approximately 2 hours. When the OD$_{600}$ reached 0.6 to 1.0, the bacteria were induced with IPTG, as described in Table 2.1 (on page 65). After induction, bacteria were pelleted by centrifugation at 3860g in an SS34 rotor (Sorvall), for 20 minutes. The supernatant was discarded and the pellets kept for protein purification.

<table>
<thead>
<tr>
<th></th>
<th>pET-3a</th>
<th>pET28a</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP-8</td>
<td>0.4mM IPTG, 5h, 37°C</td>
<td>0.4mM IPTG, 5h, 37°C</td>
</tr>
<tr>
<td>MRP-8FY</td>
<td>0.4mM IPTG, 5h, 37°C</td>
<td>0.4mM IPTG, 5h, 28°C</td>
</tr>
<tr>
<td>MRP-14</td>
<td>0.4mM IPTG, 3h, 37°C</td>
<td>0.7mM IPTG, 5h, 37°C</td>
</tr>
</tbody>
</table>

Table 2.1: Induction characteristics for various recombinant proteins in the pET3a or pET28a vectors.

2.6 Protein purification
2.6.1 pET-3a materials
Buffer A 50mM TRIS base, 2mM EDTA, 1mM DTT, pH8 at 4°C. Add 10mM MgCl$_2$, 2mM PMSF, 1mg DNase per 20ml.

Urea
Ampholine
Rotofor isoelectric focusing apparatus (BioRad #170-2910, US)
DTT
FPLC (Pharmacia, UK)
Hydroxyapatite column (BioRad, UK)

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Hydroxyapatite buffer A (HA-A) 5mM NaPO₄, 100mM NaCl, 0.02% Na₃, pH 8.
Hydroxyapatite buffer B (HA-B) 500mM NaPO₄, 100mM NaCl, 0.02% Na₃, pH 8.
Mono P column (Pharmacia, UK)
Mono P buffer A, pH 8-6 (MP-A) 25mM TRIS-acetate pH 8.3
Mono P buffer B, pH 8-6 (MP-B) Polybuffer 96 (1:13) pH 6 with TRIS-acetate

2.6.2 pET-28a materials
Charge buffer 50mM NiSO₄
Binding buffer 5mM imidazole, 0.5M NaCl, 20mM TRIS-Cl pH 7.9
Elution buffer 1M imidazole, 0.5M NaCl, 20mM TRIS-Cl pH 7.9
Strip buffer 100mM EDTA, 0.5M NaCl, 20mM TRIS-Cl pH 7.9
Hi-Trap columns (Pharmacia, UK)
Bovine Thrombin (#27-0846, Pharmacia, UK)
FPLC (Pharmacia, UK)
Hydroxyapatite column and buffers (see section 2.6.1, page 65)

2.6.3 pET-3a methods
2.6.3.1 Preparation of inclusion bodies
Bacterial pellets were resuspended in 20ml buffer A (including DNase, PMSF and MgCl₂) per litre of starting culture. Upon thawing the soup was very viscous due to the lytic release of bacterial chromosomal DNA; if this viscosity was not removed after 2 hours at room temperature (by the action of DNase) the soup was sonicated. The protein soup was then centrifuged at 22,713g (13,000 RPM in Beckman 50Ti rotor) for 20 minutes at 4°C, the supernatant discarded and the pellet dissolved in 40ml 6M urea per litre of starting culture. Dissolution was allowed to proceed overnight with gentle agitation at room temperature. Finally, dissolved protein was centrifuged for 60 minutes at 215,040g (40,000 RPM in Beckman 50Ti rotor) at room temperature and the supernatant taken for further purification.

2.6.3.2 Preparative isoelectric focusing
20ml of sample was added to 2.5ml ampholine, 1ml 1M DTT and 25.5ml 6M urea. The mix was then focused for 2 hours at 15w. Samples were harvested and visualised by coomassie stain of an SDS-PAGE gel. Protein-rich fractions were pooled and the pH
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brought back to pH 8 with 1M TRIS Base. Fractions, including precipitate were resolubilised overnight, with gentle agitation, at room temperature.

2.6.3.3 Rapid protein renaturation
Resolubilised protein was centrifuged for 30 minutes at 22,713g (13,000 RPM in Beckman 50Ti rotor) and the supernatant removed to dialysis membrane. Protein was subsequently dialysed against PBS and 2mM DTT at 4°C with very slow stirring. Dialysis solution was exchanged every 11 to 13 hours for 2 days.

2.6.3.4 Slow protein renaturation
Resolubilised protein was centrifuged for 30 minutes at 22,713g (13,000 RPM in Beckman 50Ti rotor) and the supernatant removed to dialysis membrane. Protein was subsequently dialysed against 4M urea (all solutions also contained 10mM TRIS pH 8, 1mM DTT), then 2M, 1M, 0M urea at 4°C with very slow stirring. Dialysis solution was exchanged every 11 to 13 hours.

2.6.3.5 Hydroxyapatite column separation
Sample protein was dialysed against at least 200 fold sample volume buffer HA-A, overnight, at 4°C, with gentle stirring. Sample was then loaded onto a HA-A equilibrated hydroxyapatite column, based on a FPLC. After loading, the column was washed with HA-A until the absorbance at 280nm fell to pre-loading baseline. Protein was eluted with rising concentrations of HA-B.

2.6.3.6 Cytosolic protein preparation
Bacterial pellets were resuspended in 240ml buffer HA-A (including PMSF and DTT) per litre of starting culture. Upon thawing the soup was very viscous due to the lytic release of bacterial chromosomal DNA; this viscosity was removed by sonication in 30 second bursts, continued as necessary. The protein soup was then centrifuged at 483,840g (60,000 RPM in Beckman 50Ti rotor) for 1 hour at room temperature and the supernatant filtered. This supernatant was taken for further purification.
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2.6.3.7 Mono P chromatofocusing

Sample protein was dialysed against at least 200 fold sample volume buffer MP-A, overnight, at 4°C, with gentle stirring. Sample was then loaded onto a Mono P column that had been pre-equilibrated with 3 column volumes of MP-A. Protein was eluted over 9 column volumes of 100% MP-B, based on a FPLC.

MRP-14 has a pI of 7.17 therefore it was decided to load the protein onto the column at pH 8.3, in accordance with manufacturer’s instructions. Loading at pH 8.3 gave MRP-14 a net negative charge of approximately 2.6 which allowed adherence to the column. Loading of MP-B again produced a charge gradient down the column, as it flowed through, allowing MRP-14 to deadhere when the net charge reached zero.

2.6.4 pET-28a methods

2.6.4.1 Cytosolic protein preparation

Bacterial pellets formed from induced bacteria were resuspended in 15ml binding buffer per 200ml (plus 2mM PMSF and 0.1% NP-40) of starting culture and frozen at -20°C. Upon thawing, 20ml portions of the soup were sonicated in 30 second bursts, continued until viscosity was lost (that is, as many bursts as necessary). The protein soup was then centrifuged at 215,040g (40,000 RPM in Beckman 50Ti rotor) for 20 minutes at room temperature and the supernatant filtered. This supernatant was taken for further purification.

2.6.4.2 His-tagged protein purification by nickel affinity chromatography

His-trap columns were loaded with 100ml charge buffer per column used; 1 column was used per 1L of starting culture-derived load. Columns were then preequilibrated with binding buffer and the cytosolic protein preparation loaded onto the column; flow through was collected at all stages for later analysis. After loading, columns were washed with binding buffer until the OD$_{280nm}$ reached baseline; elution was then performed via a gradient. Protein-containing fractions were evaluated by SDS-PAGE.

2.6.4.2 Thrombin assay and thrombin removal

2.6.4.2.1 Materials

S2238 substrate (Chromogenix, Italy) Stored at 10$^{-2}$M, dilute 1:10 in thrombin buffer prior to use.
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Anti-thrombin III agarose beads (Sigma, UK)
Thrombin buffer 50mM HEPES, 150mM NaCl, pH 8

2.6.4.2.2 Thrombin assay method
20μl of the protein sample to be tested for thrombin contamination and various standard concentrations of thrombin, from 0.8U/ml to 6.4U/ml were added to a 96 well plate. These wells were then supplemented with 160μl of thrombin buffer, 20μl water and finally 20μl of substrate. Plates were incubated for 10 minutes at 37°C prior to reading at 405nm in a spectrophotometer. Test sample levels of thrombin were calculated from the standards.

2.6.4.2.3 Thrombin removal
His-MRP-14 that had been initially nickel-affinity column purified was digested overnight with an average of 400U (Pharmacia units) bovine thrombin, as previously described (2.6.4.1.2, on page 68). The protein was then diluted 1:5 in water, as if to undergo hydroxyapatite selection and a sample of protein taken as a reference to assay relative thrombin contamination levels. Protein was decontaminated by the addition of 1g of anti-thrombin III agarose beads (pre-swelled and washed in 10ml PBS). The mixture was then mixed slowly at room temperature for 5 hours and again tested for thrombin contamination. Beads were removed by centrifugation at 71g (500 RPM in Beckman JS4.2 rotor) for 3 minutes. MRP-14 was then filtered through a 0.45μm filter and purified using hydroxyapatite selection.

2.6.5 Endotoxin depletion of recombinant protein
Endotoxin depletion of recombinant proteins was performed before the majority of functional protein investigations. This method is as described previously (2). Briefly, a frozen aliquot of protein and buffer control were thawed, placed on ice and Triton X-114 added to a final concentration of 0.1%. Samples were then vortexed briefly to mix the phases. After a 5 minute incubation on ice, samples were re-vortexed and warmed at 37°C for 5 minutes. Samples were then centrifuged at 9464g (13,00RPM in a microfuge) and the top phase transferred, under sterile conditions to a new Eppendorf. Finally, protein samples were assayed for protein concentration prior to use.
2.7 SDS-PAGE

2.7.1 Materials

30% acrylamide / 0.8% bis-acrylamide
10x Running buffer 250mM TRIS base, 1.92M glycine
20% SDS
10% APS
Sample buffer 2% SDS, 25% 4x stacking gel buffer, 50% glycerol, 15% H₂O
4x Stacking gel buffer 0.5M TRIS base pH 6.8
4x Separating gel buffer 1.5M TRIS pH 8.8
2 x loading buffer 2% SDS, 0.125M TRIS base pH 6.8, 25% glycerol.
Coomassie Blue stain 0.5% coomassie blue, 40% ethanol, 10% glacial acetic acid, 50% H₂O
Destain 20% methanol, 10% glacial acetic acid, 70% H₂O
Gelcode reagent (Pierce, USA)
TEMED (Sigma)
Silver staining kit (Bio-Rad, UK)
Rainbow coloured protein low molecular weight markers (#RPN 755, Amersham, UK)

2.7.2 Method

Gels were prepared as described in Maniatis (224). Briefly, acrylamide, water, separating buffer, SDS, APS and TEMED were mixed together in the order described above and were added to pre-assembled gel plates. The desired gel percentage determined the relative contribution of the components. Bubbles were removed with the addition of water saturated butanol. Once polymerisation of the separating layer had occurred, butanol was removed by washing and the stacking gel was poured. Stacking gel was produced in exactly the same way as the separating gel, except that stacking gel buffer was used. Upon polymerisation gels were run in 1 x running buffer, at 90v, until samples had concentrated at the stacking : separating gel boundary. Gels were then run at 150v until the dye front reached the bottom of the gel. Various gel stains were used including Coomassie blue, the new water based stain, Gelcode and silver stain.
### 2.8 Various cell preparations

#### 2.8.1 Materials

**2.8.1.1 WEHI 265.1 cell culture**
WEHI 265.1 monocytoid cell line (ATCC, Maryland, USA)
E4 medium (ICRF, UK)
FCS (Gibco BRL)

#### 2.8.1.2 Separation of neutrophils and peripheral blood mononuclear cells from peripheral blood

<table>
<thead>
<tr>
<th>PBS/EDTA</th>
<th>8µl 0.5M EDTA + 48µl PBS per 1ml syringe</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% dextran T500</td>
<td>6g dextran, 0.9g NaCl in 100ml H2O. Autoclave and store at 4°C.</td>
</tr>
<tr>
<td>(Pharmacia #17-0320-01)</td>
<td></td>
</tr>
<tr>
<td>100% Percoll</td>
<td>90ml Percoll + 10ml 10 x PBS makes 100ml 100%</td>
</tr>
<tr>
<td>(Pharmacia #17-0891-01)</td>
<td>Percoll. 64% made from 6.4ml 100% and 3.6ml PBS.</td>
</tr>
</tbody>
</table>

**2.8.1.3 Thioglycollate induced peritonitis derived cells**
Thioglycollate medium (BBL Becton Dickinson Microbiology Systems, USA)
PBS/EDTA 5mM EDTA in PBS

### 2.8.2 Methods

#### 2.8.2.1 Maintenance of WEHI 265.1 cells
Cells were received from ATCC with passage number unknown. Cells were thawed rapidly in a 37°C water bath and then sterilised by immersion in 70% ethanol prior to transfer to a sterile hood. Cells were then diluted in E4, 10% FCS and 0.05mM 2-ME, seeded at 1 x 10^5/ml and incubated at 37°C in 5% CO₂. Cells were checked daily and

---

<table>
<thead>
<tr>
<th>3% Stacking gel</th>
<th>15% separating gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Stacking / Separating buffer</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1ml</td>
</tr>
<tr>
<td>Water</td>
<td>6.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>40µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20µl</td>
</tr>
</tbody>
</table>
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split when they appeared dense (usually 3 days, concentration $2 \times 10^5$/ml); cells were then re-seeded at $1 \times 10^5$/ml.

When cells were used in assays, the whole culture was split. It was found that a proportion of the WEHI cells would adhere strongly to the base of the flask; therefore, to avoid selection of the adherent population by removing the non-adherent cells for assays, the total cell volume was removed and the adherent cells released with trypsin/EDTA. The cells were then pooled and re-seeded before a proportion of the total culture was then subsequently used in assays.

It was also observed that, the first day after seeding the cells into a new flask, the cells would seem more adherent to the walls of the flask and also more adherent within the adhesion assay system (increasing the noise : signal ratio). Therefore, wherever possible cells were used after 2 days in culture.

1.1.1.2 Separation of neutrophils and peripheral blood mononuclear cells from peripheral blood

Mice were sacrificed using CO$_2$ asphyxiation. Once confirmed dead, the chest cavity was opened and the heart exposed; pre-prepared 25G needle tipped, 1ml syringes containing 50µl PBS/EDTA were inserted directly above the coronary vein. After blood collection, blood was transferred to a 15ml falcon tube containing an equal volume PBS and dextran to a final concentration of 1.2%. Erythrocytes were allowed to sediment for 40 minutes.

Two Percoll solutions of 74% and 64%, in PBS, were prepared. The 64% solution was carefully layered upon the 74% solution so that an interface was visible. The plasma supernatant from the erythrocyte precipitation was added to the gradient and then the tube was centrifuged for 20 minutes at 1,138g (2,000 RPM in the JS4.2 rotor).

The fractionated components were removed in the following order; the cell free plasma and the peripheral blood mononuclear cell (PBMC) and platelet layer; the neutrophil (PMN) -rich layer found at the 64% : 74% boundary. Cells were washed out of Percoll and into assay buffer by a 5 minute centrifugation at 284g (1,000 RPM in the JS4.2 rotor) and room temperature followed by supernatant decantation and resuspension in assay buffer.
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2.8.2.3 *Thioglycollate induced peritonitis derived cells*

This method was performed as described previously (10, 152, 266). Basically, eight to twelve weeks old male C57/BL6 mice were injected intraperitoneally with 0.8ml of thioglycollate (TG) and were sacrificed by CO₂ asphyxiation 4 hours later. Migrated cells were harvested from the peritoneum by washing the peritoneal cavity with 5ml of PBS/EDTA. Migrated cells were subsequently resuspended in the assay buffer by centrifugation and resuspension.

2.9 Adhesion assays

2.9.1 Materials

- Maxisorp 96 well plates (Nunc, UK)
- Carbonate buffer 0.1M NaCO₃, pH 9.2
- 1M HEPES (sterile, tissue culture grade) (Sigma, UK)
- Human fibrinogen (Sigma, UK)
- Mouse fibrinogen (Sigma, UK)
- Keyhole limpet haemocyanin (Sigma, UK)
- 10x Hanks balanced salt solution (Gibco BRL)
- Hanks HEPES buffer 1% 1M HEPES, 10% 10 x HBSS
- 1 x cations 1mM CaCl₂, 1mM MgCl₂, 10μM ZnCl₂
- BCECF-AM (Calbiochem, USA) Stored at 10⁻²M in DMSO.
- M1/70 anti-Mac-1 monoclonal antibody
- 5C6 anti Mac-1 monoclonal antibody
- Fish skin gelatin (Sigma, UK)

2.9.2 Adhesion assay method

96 well plates were incubated at 37°C for 1 hour with 2mg/ml human fibrinogen in carbonate buffer. Wells were washed with Hanks-HEPES and pre-incubated with 25μl 4 x cations and 25μl 4x stimulant. Cells, generated and / or maintained as described above in section 2.8 (page 71), were washed into Hanks-HEPES and labelled at a density of 5 x 10⁶/ml with 1:500 1mM BCECF-AM. Washed (twice), labelled cells (50μl of 2 x 10⁶/ml) were then added to the plate and it was spun down at 70g (500RPM in Beckman plate spinning centrifuge) for 1 minute. Total fluorescence was then read at
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485nm excitation / 530nm emission and the assay was incubated at 37°C for 30 minutes. Non-adherent cells were removed by washing with Hanks-HEPES plus cations. Adhesion was calculated by reading endpoint fluorescence and expressing percentage binding by comparison with total well fluorescence.

2.9.3 Adhesion blocking experiments
Adhesion blocking experiments were carried out essentially as described above except that 12.5μl of 8 x stimulants were used instead of 25μl 4 x stimulants and 12.5μl 8 x f(Ab')2 per well were added to the BCECF-labelled cells and pre-incubated for 15 minutes prior to well addition.

2.10 Chemotaxis assays
2.10.1 Materials
3μm Transwells (CoStar, USA)
cHH Hanks-HEPES, 0.1% BSA (Sigma), 1mM CaCl₂, 1mM MgCl₂, 10μM ZnCl₂
Stimulants fMLP (Sigma, UK), IL-8 (Sigma, UK)
Modified Dunn chambers
Neuroprobe non-disposable chamber (Neuroprobe, UK)

2.10.2 Transwell chemotaxis method
3μm pore size polycarbonate Transwells were washed by pre-incubating with 1ml cHH at 37°C for an hour. Leukocytes, obtained as described above in section 2.8 (page 71), were resuspended in cHH at a concentration of 5x10⁶/ml. Assay plates were prepared by placing 600μl of cHH, containing the appropriate stimulus, in the bottom of each well. Pre-incubated Transwells had the cHH flicked off and replaced with 100μl cells. The Transwells were then added to the plates containing the bottom wells and incubated at 37°C for 2 hours. Cell migration was assessed after Transwell removal and cHH harvesting from the well bottom. Any adherent cells were removed by the addition, 5 minute room temperature incubation and harvest of PBS / 5mM EDTA. The cHH-PBS/EDTA mix was then counted on a FACS Scan and the number of migrated cells calculated.
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2.11 Calcium flux

2.11.1 Materials

Fluo-3-AM (Molecular Probes) 1μg/μl in DMSO

Flux buffer 1 x HBSS, 0.5% BSA, 1mM Ca⁺⁺/Mg⁺⁺ 10μM Zn⁺⁺

Poly-L-Lysine [PLL] (Sigma)

6cm glass bottomed dishes (#P35G-1.5-10-C, MatTek Corp., MA 01721, USA)

Pluronic F-127 (#P-2443, Sigma, UK) 15% F-127 in DMSO

2.11.2 Cuvette method

Thioglycollate cells were harvested as described in section 2.8.1.3 (page 71). Peripheral blood was taken in PBS/EDTA and the erythrocytes precipitated with 1.2% dextran for 40 minutes. The labelling method is adapted from a published method (180). Both cell types were then transferred into flux buffer by centrifugation and labelled with 1:200 Fluo-3-AM for 30 minutes at 37°C. Cells were washed once with flux buffer. 3ml of cells were then transferred to the fluorescence cuvette (containing the small flea) and the cuvette placed in the 37°C pre-warmed fluorimeter.

The fluorimeter was set at excitation 485nm / emission 530nm. Pre-treatment baselines were measured prior to treatment administration.

2.11.3 Confocal method

This method is based on a published method (180). Briefly, 6cm dishes were pre-labelled with 200μl PLL for 30 minutes at room temperature. The PLL was then flicked off and the dishes washed with 200μl flux buffer, prior to a further flicking off and air drying. Peripheral blood was separated into neutrophil-rich and mononuclear/platelet fractions as described in section 2.8.1.2 (page 71). Prepared cells were then washed into flux buffer by centrifugation and resuspension (PMN in 0.6ml / mouse used and PBMC in 3.5ml / mouse used) and 200μl of either cell preparation added to each dish. Dishes were then covered and the cells allowed to settle onto the PLL for 45 minutes.

Cells were labelled with 1μl of a 1:1 F-127:Fluo-3-AM mixture for 30 minutes prior to assaying. Dishes were placed on the confocal microscope and stimulants added via FPLC tubing, 25G needle tipped syringe and pierced lid tops.
2.12 Superoxide generation

2.12.1 Materials

H$_2$DCFDA (Molecular Probes #D-399)
cHH As described in section 2.10.1 (page 74)

2.12.2 Method

The method was carried out as per the dye manufacturer’s instructions. Briefly, PBMCs or TG-elicited cells were washed into cHH at a concentration of 1 x 10$^6$/ml and labelled with 10μM dichlorodihydrofluorescein diacetate (H$_2$DCFDA) for 20 minutes at 37°C. 5 x 10$^5$ cells were then added to 500μl stimulant and incubated for 40 minutes at 37°C prior to analysis on a FACS.

2.13 Air pouch

2.13.1 Materials

TNF-α (Sigma, UK)
Murine MIP-2 (Peprotech, USA)
LPS (Sigma, UK)
MRP-6 (M. Robinson, ICRF)

2.13.2 Method

This assay is based on a previously published method (256). Briefly, on day 0 and 3, 8 to 10 weeks old male C57/BL6 mice were anaesthetised with halothane and injected subcutaneously on the back with 2.5 ml sterile air to generate the air pouch. Sterile air was generated using a syringe driving air through a 0.22μm disposable filter. On day 6, 1 ml of stimulus was injected into the air pouch of anaesthetised mice. The stimulus was prepared in PBS containing 1mM CaCl$_2$, 1mM MgCl$_2$ and 10μM ZnCl$_2$ along with an amount of detergent-extracted HH equivalent to that present in the maximum dose of stimulant. Mice were allowed to recover and after 6 hours were sacrificed by CO$_2$ asphyxiation. Alternatively, mice were sacrificed at intervals varying from 3 to 12 hours post-injection with the stimulus. Cells were harvested from the air pouch by washing once with 1ml, then twice with 2 ml, of PBS containing 5mM EDTA. The exudate cells were counted in duplicate using a haemocytometer. Cells were then prepared for FACS analysis using standard protocols.
2.14 Cloning

2.14.1 Materials

2.14.1.1 Vectors

pAMP-1 vector kit (Gibco BRL, USA)
KS<sup>+</sup> vector (Stratagene, USA)
pZero vector (Invitrogen, Netherlands)
pET 3a vector (Novagen, USA)
pET 28a vector (Novagen, USA)

2.14.1.2 Enzymes

T4 DNA ligase (NEB, USA)
Various restriction enzymes (Boehringer Mannheim, Germany and NEB, USA)

2.14.1.3 Agarose gel electrophoresis

1 x TAE buffer 1L 50x TAE made from 242g TRIS Base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA
Ultrapure agarose (Gibco BRL)
Ethidium bromide (10mg/ml) (Sigma, UK) 1μl per 45ml of gel

2.14.1.4 DNA purification kits

Geneclean restriction fragment recovery kit
Midiprep columns (Qiagen)

2.14.1.5 Bacterial Strains

XL1-MRF<sup>+</sup> supercompetent E.coli (Stratagene)
XL1 blue competent E.coli (Originally Stratagene but also produced in house)
BL21 (DE3) pLysS competent E.coli (Stratagene, USA)
NM536 E.coli

2.14.1.6 Production of chemically competent bacteria

Solution A 60mM CaCl<sub>2</sub>, 10mM PIPES, pH to 7. Add 15% glycerol and re-pH to 7; filter sterilise.
2.14.1.7 Bacterial Media

LB medium (ICRF, UK)
BHI medium (ICRF, UK)
LB agar (ICRF, UK)

SOB (low salt) medium 2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5mM KCl, 10mM MgCl₂ pH 7.5 (autoclaved). Store at room temperature.

SOC (low salt) medium SOB, 20mM glucose (autoclaved). Store at room temperature.

LB (low salt) agar 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7 (autoclaved). Store at room temperature.

2 x TY 1% tryptone, 1% yeast extract, 0.5% NaCl (autoclaved). Store at room temperature.

2.14.1.8 Bacterial selection reagents

Ampicillin - 100μg/ml, dissolved at 33mg/ml in H₂O, stored at -20°C. (Sigma, UK)
Tetracycline - 100μg/ml, dissolved at 1mg/ml in ethanol (Sigma, UK)
Zeocin - 50μg/ml, stored at 10mg/ml and -20°C. (Invitrogen, Netherlands)
IPTG - 1mM in cloning plates, not liquid cultures, made fresh (Invitrogen, Netherlands)
Kanamycin - 33μg/ml, dissolved at 33mg/ml in H₂O, stored at -20°C. (Sigma, UK)
Chloramphenicol - 35μg/ml, dissolved at 35mg/ml in ethanol, stored at -20°C. (Sigma, UK)

2.14.1.9 Mini-preparation of plasmid DNA

Solution I 50mM glucose, 25mM TRIS Base, 10mM EDTA, pH 8. 2.5mg/ml hen egg lysozyme added immediately prior to use.
Solution II 1% SDS, 0.2N NaOH.
Solution III 60ml 5M KOAc, 11.5ml glacial acetic acid, 28.5ml H₂O (per 100ml).

2.14.1.10 Maxi-preparation of plasmid DNA

STE 100mM NaCl, 10mM TRIS base, 1mM EDTA, pH 8.
Solution I As described above in “Mini-preparation of plasmid DNA” materials.
Solution II As described above in “Mini-preparation of plasmid DNA” materials.
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Solution III  As described above in “Mini-preparation of plasmid DNA” materials.

Solution IV  10ml TE pH 8, 10g CsCl₂, 0.8ml ethidium bromide (per 10ml).
Qiagen Midiprep kit.

2.14.1.11 Screening λ-bacteriophage libraries for MRP-8 and MRP-14 homologues

129sv Phage library  129sv λ2001 library
Host Bacteria  NM536 E.coli
Top agar  50% LB medium, 50% LB agar, 10mM MgSO₄
Suspension medium  2.32g NaCl, 0.8g MgSO₄.7H₂O, 20ml 1M TRIS-HCl pH 7.5,
(SM) (per 400ml)  0.01% solid gelatin; Heat to just below boiling to dissolve
then filter sterilise
Denaturation solution  0.5M NaOH, 1.5M NaCl
Neutralisation solution  1.5M NaCl, 0.5M TRIS-HCl, pH 7.4 (with 1M TRIS-HCl
solution and 1mM EDTA for DIG)
20 x SSC  3M NaCl, 0.3M sodium citrate
Hybond N+ membranes (Amersham, UK)
Nylon membranes for colony and plaque DIG hybridisations (Boehringer Mannheim,
Germany)

2.14.1.12 Southern hybridisations of phage and plasmid DNA

2.14.1.12.1 Probe labelling with ³²P-dCTP

OLB (A:B:C mix at Freeze-thaw has no effect. Store at -20°C .
100:250:150)
Solution O  1.25M TRIS-HCl, 0.125M MgCl₂, pH 8. Store at 4°C.
Solution A  1ml solution O, 18μl 2-mercaptoethanol, 5μl dATP, 5μl
dTTP, 5μl dGTP (each dNTP dissolved in TE at 100mM).
Stored at -20°C.
TE  3mM TRIS-HCl, 0.2mM EDTA pH 7
Solution B  2M HEPES pH 6.6 with NaOH, stored at 4°C.
Solution C  Hexadeoxyribonucleotides resuspended in TE at 90 OD
units/ml. Stored at -20°C.
Various dNTPs (Pharmacia, UK)
Hexadeoxyribonucleotides (Pharmacia, UK)
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$^{32}$P-dCTP-dCTP [50μCi / 5μl] (ICN, UK)

Sephadex-G50

Klenow enzyme (Boehringer Mannheim, Germany) [2000U/ml]

2.14.1.12.2 Probe labelling with DIG

DIG High prime kit (Boehringer Mannheim, Germany)

2.14.1.12.3 Southern hybridisation with $^{32}$P-dCTP

Church hybridisation buffer (500ml) 12.1g NaH$_2$PO$_4$.2H$_2$O, 24.5g Na$_2$HPO$_4$, 35g SDS, 1ml 0.5M EDTA; heat to dissolve SDS.

2.14.1.12.4 Southern hybridisation with DIG

DIG hybridisation buffer 50% de-ionised formamide, 0.1% N-lauroylsarcosine, 7% SDS, 5x SSC, 2% blocking reagent, 50mM sodium phosphate, pH 7.0. Stored at -20°C.

Blocking reagent 10% blocking reagent in buffer 1, dissolves at 65°C. Autoclave before use. Stored at 4°C.

Buffer 1 0.1M Maleic acid, 0.15M NaCl, adjusted to pH 7.5 (20°C) with solid NaOH (7g/l) and autoclaved. Stored at 4°C.

Buffer 2 Diluted Blocking reagent solution 1:5 in buffer 1. Stored at 4°C.

Washing buffer Buffer 1 with 0.3%(v/v) Tween-20. Stored at 4°C.

Plaque Detection buffer 0.1M TRIS-HCl, 0.1M NaCl, pH 9.5

Southern Detection buffer 0.1M TRIS-HCl, 0.1M NaCl, 50mM MgCl$_2$, pH 9.5

2.14.1.13 Mini-preparations of bacteriophage DNA

DNase (Sigma, UK)

RNase (Sigma, UK)

Proteinase K (BDH, UK - 500mg 39097 1N)

Phenol (Fisons, Loughborough)

Chloroform (Sigma, UK)

$\lambda$-diluent 0.1M TRIS-HCl pH 8, 0.3M NaCl
2.14.1.14 *In vitro DNA mutagenesis*

VCS-M13 helper phage for KS\(^+\), 1 x 10\(^{11}\) pfu/ml (Stratagene, USA)

PEG/NaCl 20% PEG 6000, 2.5M NaCl

Sculptor In vitro mutagenesis kit (Amersham, UK)

5'-phosphorylated Primers (ICRF, UK)

MUMRP8-XHO1-2  \(T_m\) 67.8°C  TCTTTTCGTGACAAATGCTCGAGCCTCTG
                       GAACCTGGAG (CTCGAG is the XhoI site)

MUMRP14-BHI2  \(T_m\) 65.6°C  GAGCAAGAAGATGGGATCCGCCAACA
                       AAGCAC (GGATCC is the BamHI site)

2.14.1.15 *Generation of a LacZ-Hygromycin cassette containing plasmid*

LACZ-HYG-F  \(T_m\) 73.0°C  5'-P CTCGAGTAGTCTAGATAGTGACCGC
                       GGAGATCTGGATCCTCGAGAGCT

LACZ-HYG-B  \(T_m\) 73.0°C  5'-P CTCGAGATCCAGATCTCCGCGGTC
                       ACTATCTAGACTACTCGAGGTAC

2.14.2 *Method*

2.14.2.1 *Restriction digests of plasmid DNA*

Restriction digests were carried out according to the manufacturer’s instructions. Double digests were carried out in the Manufacturer’s recommended buffer.

2.14.2.2 *Agarose gel electrophoresis*

DNA fragments from PCR reactions or from restriction digests of plasmid or bacteriophage were diluted 1:5 in 6x loading buffer and run on an appropriate percentage agarose gel. All gels contained ethidium bromide for instant visualisation under UV light following completion of separation. Gels were run using TAE buffer at the maximum voltage permitted for the size of gel (approximately 9v/cm).

2.14.2.3 *DNA fragment purification*

After agarose gel electrophoresis, DNA fragments were excised under UV light in the minimum possible section of agarose and purified using the Geneclean kit, according to the manufacturer’s instructions.
2.14.2.4 Ligation of DNA
Approximate concentrations of DNA used for ligations were estimated from relative brightness under UV light after ethidium bromide separation and agarose gel electrophoresis. For vector fragments that had been linearised and hence ethanol precipitation purified rather than electrophoresis and Geneclean purified, concentration estimates were based on a theoretical recovery from ethanol precipitation of 90%. For 'sticky end' ligations, insert:vector molar ratios were 2:1. Ligations were carried out at room temperature for 2 hours with 0.4U T4 DNA ligase prior to transformation. If electroporation was used, DNA was ethanol precipitated, washed and resuspended in water to remove salt. If chemical transformation was to be carried out, DNA was not further purified prior to transformation.

2.14.2.4.1 Ligation of DNA using the pAMP-1 vector
This method is taken from the instructions accompanying the pAMP-1 vector kit. Primers were designed with 5'- ends containing CUA repeats and 3'- ends containing CAU repeats. PCR products were then generated and gel purified. Ligation was initiated with the addition of uracil deglycosylase (UDG) which results in abasic dUMP which can no longer pair to dAMP and reveals GAT and GTA repeats. The vector is supplied as linear DNA with GTA and CAT protruding termini. When mixed with the PCR product that has been UDG treated, rapid annealing occurs resulting in a directionally cloned vector that can then be transformed directly into competent bacteria.

2.14.2.5 Production of competent bacteria
2.14.2.5.1 Production of electrocompetent bacteria
XL-1 Blue E.coli bacteria from a glycerol stock were streaked out on a tetracycline (25µg/ml) LB plate and grown overnight at 37°C. Single colonies were picked into LB-tetracycline and grown overnight at 37°C with vigorous shaking. Bacteria were then reseeded into flasks and grown until the OD$_{595}$ reached 0.45 when they were chilled on ice for 10 minutes and then centrifuged at 2,948g (4,500 RPM in Sorvall SLA-1500 rotor) for 10 minutes at 4°C. The supernatant was discarded and the cells were suspended in ice-cold, fresh Millipore water. The centrifugation step was then repeated. Bacteria were resuspended in 10% glycerol and 90% Millipore water and re-centrifuged. Finally,
the bacteria were resuspended in 50% of the pellet volume with 10% glycerol and 90% Millipore water and frozen at -70°C.

2.14.2.5.2 Production of chemically competent bacteria

XL-1 Blue *E. coli* bacteria from a glycerol stock were streaked out on a tetracycline (25µg/ml) LB plate and grown overnight at 37°C. Single colonies were picked into LB-tetracycline and grown overnight at 37°C with vigorous shaking. Bacteria were then reseeded into flasks containing 400ml LB, plus tetracycline and grown until the OD$_{595}$ reached 0.45. Bacteria were then chilled on ice for 10 minutes and centrifuged at 2,948g (4,500 RPM in Sorvall SLA-1500 rotor) for 10 minutes at 4°C. The supernatant was discarded and the cells were suspended in 10ml ice-cold solution A. The centrifuge step was then repeated at 2,948g (4,500 RPM in Sorvall SLA-1500 rotor) for 5 minutes and the cells again resuspended in 10ml solution A. Cells were then incubated on ice for 30 minutes, re-centrifuged and finally resuspended in 2ml solution A. Cells were divided into 100µl aliquots and frozen at -70°C.

2.14.2.6 Transformation of bacteria

Most competent bacterial strains were purchased from the manufacturer and transformation was carried out exactly as specified in the accompanying instructions (this includes XL1-MRF’ and BL21 (DE3) *E. coli*).

XL1-blue bacteria were either chemically transformed or electroporated. The chemical transformation protocol is as follows; 100µl of bacteria were incubated with 50ng of DNA and incubated on ice for 10 minutes. Bacteria were then incubated at 37°C for 5 minutes, and then had 1ml of media added. Bacteria were then shaken for 1 hour at 37°C before being plated out on plates containing the appropriate selection.

The electroporation transformation protocol is as follows; 40ul of bacteria were incubated with 50ng of DNA (which contained no salts) in a 0.1 cm cuvette and electroporated at 1.8kV, 200 ohms and 25mF. Immediately after transformation, bacteria had 1ml of media added (minus antibiotics) and were shaken for 1 hour at 37°C before plating out on plates containing the appropriate antibiotic.
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2.14.2.7 Mini-preparation of plasmid DNA

This method is a shortened version of the standard method as used in Maniatis (224). Basically, 1ml of bacteria from an overnight culture were sedimneted by subjecting to 9,464g for 30 seconds (13,000RPM) in a microfuge. The supernatant was discarded and the bacterial pellet resuspended in 100μl of solution I. After a 5 minute room temperature incubation, 200μl of solution II was added and the tubes shaken. 150μl of solution III was then added and the tubes again shaken until precipitation of bacterial chromosomal DNA was complete. The cell membrane and chromosomal DNA pellet was sedimented by a 3 minute centrifugation step at 9,464g (13,000RPM). The plasmid DNA containing supernatant was then transferred to a new tube and precipitated by the addition of 900μl 100% ethanol and mixing. Plasmid DNA was pelleted after a 5 minute room temperature incubation by a 10 minute centrifugation at 9,464g (13,000RPM). The ethanol-salt supernatant was discarded and the plasmid DNA/RNA pellet was resuspended in 100μl of water. 6μl or 15μl was subsequently used for restriction analysis, if a high or low copy number plasmid was present in the prepared bacteria, respectively.

2.14.2.8 Maxi-preparation of plasmid DNA

Two methods were used for large scale plasmid preparations; the caesium chloride banding method and the Qiagen column purification method. The Qiagen column purification method was performed exactly as described in the manufacturer's instructions. Details of the caesium chloride banding method is detailed below.

Starting culture volume for preparation was dependent on the plasmid copy number. For high copy number plasmids such as pZero or KS+, 500ml was used. For low copy number plasmids such as the pET vectors, 2L was used.

2.14.2.8.1 Caesium Chloride banding

Each step in the following protocol refers to 200ml of starting culture volume as the unit to which steps have been applied. An overnight culture was pelleted by centrifuging at 2,150g (4,000RPM in the Sorvall SS34 rotor) for 20 minutes. The bacterial pellet was then washed twice in 200ml 4°C STE. After washing, the pellet was resuspended in 8ml solution I and the suspension incubated for 5 minutes at room temperature. Next, 8ml of solution II was added. Complete bacterial lysis was ensured by mixing and
incubating for 10 minutes at room temperature. Cell membranes and chromosomal DNA were precipitated by the addition of 6ml of solution III, mixing and incubating for 30 minutes on ice. The precipitate was then cleared by centrifugation at 13,440g (10,000RPM in the Sorvall SS-34 rotor) for 20 minutes. The supernatant was then transferred into a new tube containing 0.7 volumes of propan-2-ol, mixed and incubated at room temperature for 30 minutes. The plasmid DNA was pelleted by centrifugation at 9,710g (8,500RPM in the Sorvall SS-34 rotor) for 8 minutes and the pellet washed in 2ml 70% ethanol. The washed pellet was subsequently re-centrifuged, the supernatant removed and the pellet air-dried under flow-hood conditions for 1 hour.

Plasmid DNA was prepared for caesium banding by dissolving the dried DNA pellet in 10ml solution IV. Any precipitate was cleared by centrifugation at 1,210g (3,000RPM in the SS-34 rotor) for 10 minutes. Supernatant was then transferred to ultra-centrifuge tubes and centrifuged at 322,560g (60,000RPM in a Beckman 70Ti rotor) for 15 hours. After centrifugation, 3 bands were usually seen; the top band was chromosomal DNA, the middle was plasmid DNA and the bottom smeared band was RNA. Plasmid DNA was removed using a 19G needle and 5ml syringe; ethidium bromide was removed from the plasmid DNA by a number of washes in water saturated butanol. Butanol was added to ethidium bromide labelled plasmid DNA and the mixture inversion mixed and centrifuged to separate the phases. The pink butanol phase was removed and stored for safe disposal.

Finally, plasmid DNA was diluted 1:2 in water to prevent CsCl₂-DNA co-precipitation and was precipitated with 2 volumes of 100% ethanol. The resultant pellet was washed with 70% ethanol, air dried and dissolved in TE. DNA concentration was estimated from the OD₂₆₀.

2.14.2.9 Screening λ-bacteriophage libraries for MRP-8 and MRP-14 homologues

2.14.2.9.1 Preparation of λ-phage host bacteria, NM536

NM536 bacteria were prepared as a host strain for infection with λ-bacteriophage by seeding a small portion of a -70°C glycerol stock into LB medium, supplemented with 0.2% maltose and growing overnight. Bacteria were subsequently pelleted by centrifugation @ 2,150g (4,000RPM in a SS-34 rotor) for 10 minutes. The LB supernatant was then discarded and the bacteria resuspended in 20ml of sterile 10mM MgSO₄ at a concentration of 1.6 x 10⁹/ml (measured by correcting OD₆₀₀ to 2). NM536
were subsequently stored at 4°C for a maximum of 3 weeks. This method is as described in Maniatis (224).

### 2.14.2.9.2 Preparation of λ-phage libraries

In order to maximise plaque density without causing confluence and to prevent screening of numerous blots by having a low plaque density, λ-phage was titrated onto a NM536 agar plate. Once the correct dilution for the particular batch of λ-phage had been determined, a library of λ-phage was then plated out in order to represent the entire 129 mouse genomic library (4 x 506.25 cm² plates, in this case).

506.25 cm² (square plates with 22.5 cm sides) L-agar plates were prepared under sterile conditions, without selection and allowed to dry (this prevents top agar from peeling off during filter transfer). 1ml of NM536 were mixed with approximately 118,000 λ-phage (125 μl) and incubated at 37°C for 20 minutes. NM536/λ-phage were then mixed with 20ml top agar which had been kept molten at 47°C and poured onto the plate. Once the top agar had hardened, plates were inverted with filter paper placed in the lids (to absorb condensation) and the plates were incubated at 37°C for 10 to 12 hours, or to the point at which plaques were about to make contact but were non-confluent (λ-phage undergo recombination so confluence must be avoided). Chloroform laden cotton wool was then added to plate lids, to prevent further bacterial growth, and the plates were chilled at 4°C for an hour (to harden the top agar but also to prevent bacterial growth).

Plates were inverted so that the plaques were face up and 400 cm² (20 cm square) nylon filters (type varied according to hybridisation method used) were placed on top of the plaque-rich top agar for 1 minute. Filter positions were marked with permanent marker and the filter was then carefully removed and placed in denaturation solution for 5 minutes. The filter was then transferred into neutralisation solution for 15 minutes before rinsing in 2 x SSC and drying. Finally, DNA was fixed to the membranes by either baking at 80°C for 30 minutes (Hybond-N+) or UV cross-linking in a Stratalinker (BM neutral nylon membrane).

A second filter for each plate was then prepared to eliminate any false positives from the hybridisation results. This method is as described in Maniatis (224).
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2.14.2.9.3 Isolation of single, positive λ-phage

After the initial screen, any areas of the original λ-phage-bacteria plate containing positively-identified λ-phage were picked, using a 200μl tip into a 1.5ml Eppendorf containing 2ml SM medium (this usually was between 10 and 50 phage). This was incubated overnight at 4°C to allow diffusion of λ-phage out of the plug before making logarithmic dilutions from 1:10 up to 1:1 x 10^6 and plating onto circular, 10cm Petri dishes. This was then followed by other rounds of selection by hybridisation before a single, isolated λ-phage clone could be picked.

2.14.2.10 Southern hybridisations of phage and plasmid DNA

Probes to be labelled were prepared from Geneclean purified, agarose gel separated, restriction digests or PCR reactions. Calculations of hybridisation temperatures were based on the equation

\[ T_m (°C) = 81.5 + 16.6 \log_{10} M + 0.41(\%GC) - 0.61(\%formamide) - \frac{500}{n}, \]

taken from (179).

\( M = \) concentration of monovalent cation, in moles.
\( n = \) probe length in bases

2.14.2.10.1 Probe labelling with \( ^{32}P \)

This method is based on a published method (72). The following components were added to a screw-top 1.5ml Eppendorf in the following order; (1) 50ng DNA (as estimated by agarose gel intensity) up to 32.5μl, which was denatured by heating at 94°C for 5 minutes and then placing on ice (2) water, to 32.5μl (3) 10μl OLB (4) 2μl 10mg/ml BSA (5) 5μl \( ^{32}P \)-dCTP (6) 2 units of Klenow polymerase. Probes were labelled overnight at room temperature.

Labelled DNA was separated from un-incorporated \( ^{32}P \)-dCTP using a previously described method (9). Briefly, disposable spin-columns were prepared as follows; 1ml syringes (plunger removed) were plugged with glass wool and filled with Sephadex-G50 beads (swelled, washed and stored in TE). Columns were then placed within Falcon 15ml tubes, containing a 1.5ml Eppendorf with the top removed, and centrifuged at 640g (1,500RPM in the JS4.2 rotor) for 3 minutes. The Eppendorf was then replaced before the probe was added to the top of the column and the column re-centrifuged. All the free label remained on the column whereas the labelled probe flowed through and was collected in the Eppendorf.
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2.14.2.10.2 Probe labelling with DIG

Template DNA and water were added to a total volume of 16µl in a 1.5ml Eppendorf and denatured by incubating at 94°C for 10 minutes and transferring to ice. 4µl of DIG-High prime was then added and mixed. The labelling reaction was allowed to proceed for 20 hours at 37°C before the reaction was terminated by heating at 65°C for 10 minutes.

2.14.2.10.3 Southern hybridisation with \textsuperscript{32}P-dCTP

This method is based on the standard method, as described in Maniatis (224). Briefly, pre-prepared filters were wetted in 2 x SSC and placed within a 25cm hybridisation tube (no more than 4 per bottle). The 2 x SSC was drained and replaced with 20ml Church buffer. The filters were then pre-hybridised for an hour at 65°C. Purified probe was denatured by heating at 94°C for 5 minutes followed by incubation on ice. The probe was then added to the hybridisation vessel and the hybridisation reaction allowed to proceed overnight at 65°C.

Post-hybridisation stringency washing was performed as follows; the probe was poured off and the filters rinsed with 2 x SSC 0.1% SDS, 5 minutes wash with room temperature 2 x SSC 0.1% SDS at 65°C; two 15 minute washes with 65°C 2 x SSC 0.1% SDS at 65°C; one 20 minute wash with 65°C 0.1 x SSC 0.1% SDS at 65°C. Washed filters were exposed overnight at -70°C.

2.14.2.10.4 Southern hybridisation with DIG

This method was developed after some difficulties with the manufacturers standard method and is very similar to that used for Southern hybridisation with \textsuperscript{32}P-dCTP. Blots were pre-hybridised at 55°C with 20ml per 100cm\textsuperscript{2} of hybridisation solution.

Purified probe was denatured by heating at 94°C for 5 minutes followed by incubation on ice. The probe was then diluted at 1µl per ml in hybridisation solution and was used as a replacement for the hybridisation solution, with a minimum volume of 2.5ml per 100cm\textsuperscript{2} and at least 12ml per large hybridisation bottle. The hybridisation reaction was then allowed to proceed overnight at 55°C.

Post-hybridisation stringency washes were as follows; the probe was poured off and the filters rinsed with room temperature 2 x SSC 0.1% SDS, then an 8 minutes wash with 50ml 55°C 2 x SSC 0.1% SDS at 55°C; finally, two 24 minute washes with
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55°C 0.1 x SSC 0.1% SDS at 55°C. Washed filters were then developed, analogously to a Western blot, as described below.

Filters were transferred to sandwich boxes and washed with washing buffer for 5 minutes. Membranes were then blocked with 100ml per 100cm² blocking buffer for an hour and probed with 20ml per 100cm² anti-DIG-AP antibody at 1:10,000 (or 1:20,000 when CSPD-Star was used), diluted in buffer 2 for 30 minutes. Filters were then washed twice in washing buffer for 15 minutes and equilibrated in the appropriate detection buffer for 5 minutes. DIG hybridisation was visualised with the addition of 1:100 CSPD or CSPD-Star, diluted in detection buffer and incubated at room temperature for 5 minutes. Blots incubated with CSPD were incubated at 37°C for 15 minutes prior to exposure to film.

2.14.2.11 Mini-preparations of bacteriophage DNA

The plaque containing the λ-phage of interest was seeded into 50ml LB containing 10mM MgSO₄ and NM536 bacteria and grown overnight at 37°C. When evidence of bacterial lysis was visible (white residue), bacteria were killed with the addition of 300μl chloroform followed by incubation for 5 minutes at 37°C, with shaking. The λ-phage soup was then centrifuged for 20 minutes at 840g (2,500rpm in a SS-34 rotor) and the supernatant transferred to a new tube containing DNase and RNase, both to a final concentration of 1μg/ml. 1ml of soup was kept as a phage stock for further inoculations. After a room temperature incubation of 30 minutes, the solution was centrifuged for 2 hours at 34,406g (16,000RPM in a SS-34) leaving a brown pellet of λ-phage; the supernatant was discarded. The λ-phage pellet was resuspended in 300μl λ-diluent and transferred to a Eppendorf tube containing 7μl 0.5M EDTA to disrupt the phage capsule and release the phage DNA. After mixing, SDS was added to a final concentration of 0.5%. Protein (phage capsule) lysis was initiated by the addition of 50μl proteinase-K (10mg/ml) and allowed to continue at 56°C for 30 minutes.

DNA was separated from protein contaminants by extractions with phenol, phenol : chloroform and chloroform and precipitated by the addition and mixing of 2 volumes of ethanol. Finally, the resultant pellet was washed with 70% ethanol and dissolved in 100μl TE.
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2.14.2.12 In vitro DNA mutagenesis

2.14.2.12.1 Preparation of single stranded DNA

The area of DNA to be mutagenised was cut down (if necessary) to 3kb and subcloned into the KS* vector. Bacteria carrying the vector were grown up to OD_600 0.5 in 2 x TY broth plus 1% glucose and 100μg/ml ampicillin and then infected with the 1 x 10^{10} pfu VCS-M13 helper phage. Infection was optimised by incubation without shaking for 45 minutes, followed by a shaking incubation for 45 minutes. Bacteria were then pelleted and resuspended in new 2 x TY broth with ampicillin and kanamycin (25μg/ml) selection, to select for phage and plasmid, double positive bacteria and then grown overnight at 37°C.

Single stranded DNA (ssDNA) was prepared as follows. Bacteria were pelleted and the supernatant taken into 20% PEG/NaCl and 80% supernatant; the mixture was then incubated on a wheel at 4°C for 60 minutes. ssDNA was pelleted by centrifugation at 13,440g (10,000RPM in a SS-34) for 20 minutes at 4°C and subsequently dissolved in 1ml TE. ssDNA was then extracted with phenol, 1 : 1 phenol : chloroform and chloroform, and precipitated with the addition of 100μl 3M NaOAc (pH 5.2) and 2.5ml 100% ethanol. Finally, ssDNA was pelleted by centrifugation at 19,354g (12,000RPM in a SS-34), washed with 70% ethanol, dried down and dissolved in 1ml TE. This method is as described in Maniatis (224).

2.14.2.12.2 In vitro DNA mutagenesis

This method is taken from the Sculptor kit instructions. Briefly, 10pM 5'-phosphorylated primer was annealed to 2μg ssDNA by heating the mixture to 95°C and allowing to cool to 37°C by placing in a 37°C bath. A mutant strand was then synthesised using T7 DNA polymerase and T4 DNA ligase, and incubating the mixture at 37°C for 30 minutes. Enzymes were then inactivated by heating at 70°C for 15 minutes. Any remaining ssDNA was subsequently removed by incubation with T5 exonuclease at 37°C for 30 minutes; this enzyme was also inactivated by a 15 minute, 37°C incubation.

The original strand was then nicked with NciI for 90 minutes at 37°C and then digested for 30 minutes at 37°C with exonuclease III. Both enzymes were then inactivated by heating the reaction at 70°C for 15 minutes. Mutant strands were insensitive to NciI nicking because the dNTPs use to synthesise the strand contain a
dCTP which has a sulphur for oxygen substitution in the α-phosphate moiety; this leads to non-recognition of the mutant strand by NcIⅠ.

A second mutant strand was then synthesised using standard dNTPs, DNA polymerase I and T4 DNA ligase, incubated at 37°C for 60 minutes. Finally, mutant plasmid was transformed into supercompetent bacteria.

2.14.2.13 Generation of a LacZ-Hygromycin cassette containing plasmid

The primers described above, in section 2.14.1.15 (on page 81), were used to replace the MCS of KS⁺. On this base, the LacZ-Hygromycin plasmid was built using standard cloning methods as described in the preceding sections.

2.15 ES cell maintenance and transfection

2.15.1 Materials

2.15.1.1 Construct purification and linearisation

5'-phosphorylated Hairpin oligonucleotide (ICRF, UK)

NOT1-HAIRPIN  Tm 78.3°C  GGCCTCCGGTACATGATCGAGGGACTGACA
AGACGGCCAGTCCTCGATCATGTACCGGA

T4 DNA ligase

Sephacryl S-400 size select column (Pharmacia, UK)

2.15.1.2 ES cell DNA preparation and Southern blotting

ES cell lysis buffer  10mM TRIS pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% sarcosyl. Proteinase K was added at a final concentration of 1mg/ml prior to use.

Restriction buffer  1 x restriction buffer, 1mM spermidine, 100µg/ml BSA,
100µg/ml RNase and 30U restriction enzyme.

Depurination buffer  0.25N HCl

Denaturation buffer  1M NaOH, 1.5M NaCl
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2.15.2 Method

1.1.1.1 ES cell DNA preparation and Southern blotting

DNA was ethanol precipitated and digested overnight as described previously (212). Briefly, ES cells grown to confluence were washed out of medium with PBS and were digested with 50 μl ES cell lysis buffer overnight at 60°C in a humid atmosphere. 100 μl of precipitation buffer was then added to each well and the precipitation reaction allowed to proceed for 30 minutes at room temperature. Samples were carefully washed; supernatant was carefully removed by inverting the plate and decanting the contents onto paper towels inserted between the plate and the lid. Samples were then washed 3 times with 150 μl of 70% ethanol, dripped vertically onto the wells when the plate was held at 45°. Samples were then allowed to dry before digestion.

Each sample received 30 μl of restriction buffer which was mixed as well as possible, in order to dissolve the dried DNA and was digested overnight at 37°C in a humid atmosphere. Loading buffer was then added to samples which were then electrophoresed in 0.7% agarose TAE gels in TAE at 100v for 4 hours. Gels were photographed under UV illumination, depurinated for 12 minutes with depurination buffer, denatured for 30 minutes and blotted overnight in denaturation buffer onto Hybond N+ membranes. Membranes were then washed in 2xSSC, air dried and then cross-linked for 30 minutes at 80°C.

Membranes were then hybridised as described above with ³²P-dCTP-labelled probes and washed and exposed as described above. Recombination events were scored from the resultant films.

2.15.2.2 Construct purification and linearisation

Both MRP-8 and MRP-14 knockout constructs were maxi-prepped using the Qiagen midiprep columns (as described in section 2.14.2.8). Purified DNA was linearised by overnight incubation of 140 μg DNA with 140U NcoI. DNA was subsequently purified with a 1:1 phenol: chloroform extraction and ethanol precipitation before it was finally dissolved in water. DNA was quantitated using a spectrophotometer and the concentration altered to 1 mg/ml, prior to freezing at -20°C.

After linearisation, some constructs were capped with a hairpin oligonucleotide. Ligation was performed using a standard overnight ligation using a molar ratio of hairpin: construct of 100:1 to prevent construct recircularisation. The construct was
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subsequently purified from the linker by use of a size select spin column. The column
was equilibrated with 2ml 1x STE, then 2ml 0.66 x STE followed by the construct and 2
volumes of ligation mix of 1 x STE. The construct was purified by a 410g (1,200RPM
in a JS4.2 rotor). Finally, the construct was 1 : 1 phenol : chloroform extracted and
ethanol precipitated, prior to being dissolved in water and frozen at -20°C.

2.15.2.3 ES cell maintenance and transfection
ES cells were maintained in accordance with the previously published method (212).
Standard ES cell culture reagents and antibiotics were used. All cell culture and
transfection was performed at Clare Hall in the laboratory of Ian Rosewell.

Various ES cell lines were used. For the 2 MRP-8 transfections and the first
MRP-14 transfection, the GK129 (derived from 129 Ola strain mice) was used. The cell
line used for the second MRP-14 transfection was a clone of GK129, 129/1 (essentially
the same and also 129 Ola derived). All fibroblasts that were used as feeder cells were
derived from day 13/14 embryos.

2.16 Genotyping by Southern hybridisation and PCR

2.16.1 Materials

2.16.1.1 Preparation of DNA from mouse tails
Tail lysis buffer 50mM TRIS base, 100mM EDTA, 100mM NaCl, 1% SDS
1mg Proteinase K / 2.5ml lysis buffer added prior to use

2.16.1.2 Southern hybridisation of samples to be genotyped
Tail restriction buffer 15µl (from 50µl total) tail DNA, 3µl 1 x restriction buffer,
3µl restriction enzyme (10U/ml), 9µl RNase.
Depurination buffer As described in section 2.15.1.2.
Denaturation buffer As described in section 2.15.1.2.

2.16.1.3 PCR genotyping of MRP-8 and MRP-14 knockout mice
1 x PCR buffer As described in section 2.2.1

Primers
G-Neo  Tm 56.2°C ACGCTTCCCTCGTGCTTTACG
14G-GF1 Tm 50.3°C AACATCTGTGACTCTTTAGCC
2.16.2 Method

1.1.1.1 Preparation of DNA from mouse tails

The end 1 to 2cm of tail from newly weaned mice (approximately 21 days) was cut off and transferred into a 1.5ml Eppendorf containing 700μl lysis buffer. Lysis reactions were allowed to proceed overnight at 56°C.

DNA was extracted from the tail lysate using the following method. 700μl of 1 : 1 phenol : chloroform was added to the lysate and shaken vigorously on a yeast homogeniser for 10 minutes. After mixing, the lysates were centrifuged at 9,464g (13,000RPM in a microfuge) for 10 minutes to efficiently separate the organic and aqueous phases. The upper, aqueous phase was removed to a new 1.5ml Eppendorf and the DNA precipitated by the addition of 0.7 volumes of propan-2-ol. The DNA was then pelleted by a 10 minute centrifugation at 9,464g (13,000RPM in a microfuge). The resultant, small pellet was then carefully washed with 70% ethanol, air dried and dissolved in 50μl water.

2.16.2.2 Southern hybridisation of samples to be genotyped

Once DNA had been digested, using tail restriction buffer, genotyping by Southern hybridisation was carried out essentially as described above in section 2.15.2.1.

2.16.2.3 PCR genotyping of MRP-8 and MRP-14 knockout mice

This method is essentially the same as that described in section 2.2.2. Reactions were set up with 25μl 1 x PCR buffer containing the 3 primers 14G-GF1, 14G-GB1 and G-Neo, (for MRP-14 genotyping) and 8G-GF1, 8G-GB2 and G-Neo (for MRP-8 genotyping) with 1μl DNA. Cycling was as follows; 94°C for 5 minutes, then 32 cycles of 94°C for 20 seconds, 48°C for 60 seconds and 72°C for 60 seconds, finally, 72°C for 5 minutes. PCR products were then run on a 2% agarose gel and the products resolved over 10cm. MRP-14 genotyping results were, wild type mice (WT) had a single band of 380 base pairs (bp), heterozygote mice (HET) had 2 bands of 380bp and 100bp, whereas knockout mice (KO) had a single band of 100bp. MRP-8 genotyping results
were, WT mice had a single 535bp band, HET mice had two bands of 535bp and 575bp whereas KO mice had a single 575bp band.

2.17 Fluorescent sequencing and sequence analysis

2.17.1 Fluorescent sequencing materials

PCR tubes
Ampli TAQ DNA polymerase and fluorescent dye terminator mix
Precipitation buffer (per 52μl) 2μl 3M NaOAc pH 4.6, 50μl 95% ethanol
Primer

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<tr>
<td>7.4T7F3</td>
<td>62.4°C</td>
<td>8/10/98</td>
<td>CAGACAGTGGAGGGCAGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAAC</td>
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</tbody>
</table>
Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>T_m</th>
<th>Date</th>
<th>Sequence</th>
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<tr>
<td>7.4T7B3</td>
<td>58.4°C</td>
<td>8/10/98</td>
<td>CACCTGTAGACACCTGGTGGTC</td>
</tr>
<tr>
<td>14B2F3</td>
<td>58.7°C</td>
<td>8/10/98</td>
<td>CCAGCAGCTGTGTAGTACTGAG</td>
</tr>
<tr>
<td>14F2B</td>
<td>60.9°C</td>
<td>13/8/98</td>
<td>GGGCTGTTTATCACTCAGACCTGG</td>
</tr>
<tr>
<td>14B2F</td>
<td>54.2°C</td>
<td>13/8/98</td>
<td>CACAGGGAGTGCTTAGATAGC</td>
</tr>
<tr>
<td>7.4T7F</td>
<td>54.7°C</td>
<td>13/8/98</td>
<td>GCATAACCAGCTATAGCTCTCCAT</td>
</tr>
</tbody>
</table>

Other primers were synthesised and used in the effort to sequence MRP-14. These primers either did not produce good quality data or did not prime at all; for these reasons, they are not represented here.

2.17.2 Fluorescent sequencing method

0.5μg or 1μg DNA was added to PCR tubes and supplemented with 170ng of the sequencing primer, 8μl terminator mix, and water, up to a total volume of 20μl. Cycling was performed as described below.

\[
\begin{align*}
5' @ 96°C & \\
10'' @ 96°C & \\
5'' @ (T_m -3)°C & 25 cycles \\
4' @ 60°C & \\
\text{Store @ 15°C} &
\end{align*}
\]

Reactions were prepared for gel electrophoresis by transferring to a 1.5ml Eppendorf containing 52μl precipitation buffer, mixing and incubating on ice for 10 minutes. Precipitated PCR products were pelleted by centrifuging for 30 minutes at 4°C and 9,464g (13,000RPM in a microfuge) and subsequently washed with 250μl 70% ethanol. Pellets were again centrifuged at 9,464g for 10 minutes at 4°C prior to air drying for 10 minutes at room temperature. Pellets were then stored at -20°C prior to gel electrophoresis and computer analysis.
2.17.3 Sequence analysis materials and methods

<table>
<thead>
<tr>
<th>Program used</th>
<th>Function</th>
<th>Web address</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RepeatMasker2</td>
<td>Repeat identification</td>
<td><a href="http://ftp.genome.washington.edu/">http://ftp.genome.washington.edu/</a></td>
<td>(239)</td>
</tr>
<tr>
<td>BLASTX 2.0.6</td>
<td>Protein translation</td>
<td>ICRF intranet</td>
<td>(6)</td>
</tr>
<tr>
<td>Promoter</td>
<td>Promoter prediction</td>
<td><a href="http://www-identification.hgc.lbl.gov/promoter.html">http://www-identification.hgc.lbl.gov/promoter.html</a></td>
<td>(214)</td>
</tr>
</tbody>
</table>

The final sequence was submitted to the above programs and the subsequent data collected and analysed.

2.18 RT-PCR

2.18.1 Materials

2.18.1.1 RNA isolation
TRIzol reagent [Total RNA isolation reagent] (Gibco BRL)
Diethyl pyrocarbonate [DEPC] (Sigma, UK)
ART filter tips (Merck-MBP, UK)

2.18.1.2 RT-PCR
RT-PCR kit (Stratagene, USA)

1 x PCR buffer As described in 2.2.1

Primers C8F, C8B, C14F, C14B As described in section 2.4.1
8F1, 8B2, 14F2, 14B2 As described in section 2.2.1
HPRT F Tm 52.2°C GCTGGTGAAAAGGACCTCT (84)
HPRT B Tm 54.5°C CACAGGACTAGAACACCTGC (84)

2.18.2 Method

2.18.2.1 RNA isolation
This method is taken from the instructions accompanying the TRIzol reagent. TRIzol is a monophasic reagent comprised of phenol and guanidine isothiocyanate. The reagent was added directly to cells (1ml per 5 x 10^6 cells) and incubated at room temperature to lyse the cells completely. 200μl of chloroform was then added and the samples
centrifuged at 9,464g (13,000RPM in a microfuge) for 15 minutes at 4°C to generate aqueous and organic phases; the RNA was recovered from the upper, aqueous phase and subsequently precipitated with propan-2-ol. RNA was then pelleted by centrifugation at 9,464g for 10 minutes at 4°C. Finally, the RNA pellet was washed in 75% ethanol, centrifuged at 7500g for 5 minutes at 4°C, air dried and dissolved in DEPC-treated water at 60°C.

2.18.2.2 RT-PCR

This method is taken from the instructions accompanying the RT-PCR kit. RNA produced using the TRIzol reagent was quantitated spectrophotometrically and 5μg of total RNA was used in the cDNA synthesis. Briefly, the first strand of cDNA was synthesised using 50U MMLV-RT and dNTPs in the presence of RNase inhibitor, at 37°C for 1 hour. The RT was then deactivated by a 5 minute incubation at 90°C. Varying amounts of the cDNA were then used in a standard PCR reaction with cycling profiles as detailed below. PCR products were then run on a 2% agarose gel and the products resolved over 10cm.

<table>
<thead>
<tr>
<th>Primers</th>
<th>c8F/c8B</th>
<th>c14F/c14B</th>
<th>8F1/8B2</th>
<th>14F2/14B2</th>
<th>HPRT F /HPRT B</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' @ 94°C</td>
<td>90&quot; @ 94°C</td>
<td>90&quot; @ 94°C</td>
<td>20&quot; @ 94°C</td>
<td>20&quot; @ 94°C</td>
<td>20&quot; @ 94°C</td>
</tr>
<tr>
<td>90&quot; @ 50°C</td>
<td>90&quot; @ 50°C</td>
<td>30&quot; @ 55°C</td>
<td>30&quot; @ 55°C</td>
<td>30&quot; @ 58°C</td>
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</tr>
<tr>
<td>60&quot; @ 72°C</td>
<td>60&quot; @ 72°C</td>
<td>60&quot; @ 72°C</td>
<td>60&quot; @ 72°C</td>
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</tr>
<tr>
<td>5' @ 72°C</td>
<td>5' @ 72°C</td>
<td>5' @ 72°C</td>
<td>5' @ 72°C</td>
<td>5' @ 72°C</td>
<td></td>
</tr>
</tbody>
</table>

2.19 FACS staining and analysis

2.19.1 Materials

Extracellular and intracellular staining

FACS wash Per 400ml : 0.8g BSA (Sigma), 4ml normal goat serum (Sigma), 0.4g NaN₃, made in PBS

FACS fix Per 90ml : 85ml PBS, 5ml 38% formaldehyde

Flexiwells - PVC microtiter "U" bottomed plates (Dynex Technologies, Chantilly, VA, USA)

FACS tubes - 5ml round bottom polystyrene tubes (Becton Dickenson, France)
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**Various primary antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody name</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>CD3 (KT3), IgG2b ? (ICRF)</td>
<td>Tissue culture supernatant used neat.</td>
</tr>
<tr>
<td>MRP-8</td>
<td>NH-7, rabbit polyclonal (ICRF)</td>
<td>Used at 1:400 in FACS wash</td>
</tr>
<tr>
<td>MRP-14</td>
<td>NH-6, rabbit polyclonal (ICRF)</td>
<td>Used at 1:400 in FACS wash</td>
</tr>
<tr>
<td>MRP-14</td>
<td>2B10, rat monoclonal, unknown isotype (ICRF)</td>
<td>Tissue culture supernatant, 10μl used neat plus 90μl FACS wash</td>
</tr>
<tr>
<td>Neutrophils*</td>
<td>7/4, IgG2a, (gift from S.Gordon, Oxford, UK)</td>
<td>Tissue culture supernatant, 50μl used neat plus 50μl FACS wash</td>
</tr>
<tr>
<td>Macrophages / Monocytes**</td>
<td>F4/80, rat monoclonal, IgG2b, (gift from S.Gordon, Oxford, UK)</td>
<td>Used at 1:100 in FACS wash</td>
</tr>
<tr>
<td>IgG2bk</td>
<td>IgG2bk, (Pharmingen)</td>
<td>Dependent on antibody being controlled</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

- FITC conjugated goat anti-rat IgG (Jackson Immunoresearch, USA) | Used at 1:200 in FACS wash |
- FITC conjugated goat anti-rabbit IgG (Sigma) | Used at 1:160 in FACS wash |

**Intracellular staining**

- FACS permeabilisation buffer | FACS fix, 0.4% Triton-X 100 |

* 7/4 recognises a 40kDa antigen found predominantly on neutrophils but also on activated macrophages (83, 99).

**F4/80 recognises a 150kDa antigen found mainly on mature macrophages but also in low amounts on monocytes (12, 83).

### 2.19.2 Extracellular FACS staining method

Dependent on cell availability, between $1 \times 10^5$ and $1 \times 10^6$ cells were added to each well of a flexiwell plate, one well per antibody stain, and embedded in a standard 96 well plate. Cells were washed into FACS wash by pelleting, flicking off the supernatant and resuspending the cells in the dead volume by vortexing the plate (this defines “washing”). Pelleting was achieved by centrifuging the plate at 224g (1,000 RPM in Beckman plate spinning centrifuge).
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To the dead volume was added to 200μl 4°C FACS wash. Cells were washed and resuspended in either 90μl FACS wash plus 10μl of primary antibody at the appropriate dilution or in primary antibody, if it was a tissue culture supernatant, such as CD3. Cells were then incubated on ice for 20 minutes and then washed 3 times with 200μl FACS wash. To the resuspended cells was added 100μl of secondary antibody. Cells were then incubated on ice for 20 minutes and washed 3 times with 200μl FACS wash.

If cells were to be analysed immediately, they were resuspended in 200μl 4°C FACS wash and transferred to FACS tubes. Otherwise, cells were resuspended in 200μl 4°C FACS fix and stored under foil at 4°C until analysis.

2.19.3 Intracellular FACS staining method

This method was adapted by Jo Porter (ICRF). Briefly, between 1 x 10^5 and 1 x 10^6 cells were added to each well of a flexiwell plate and embedded in a standard 96 well plate. Cells were washed into 100μl FACS fix and incubated for 20 minutes on ice. 100μl of FACS permeabilisation buffer was then added to the cells and they were again incubated for 20 minutes on ice. Cells were then washed 3 times with 200μl FACS wash before staining and preparing for analysis exactly as described in the preceding section.

2.20 Immunoaffinity column purification

2.20.1 Materials

IA carbonate buffer 0.5M NaCl, 0.1M NaCO₃, pH 9.2

IA acetate buffer 0.5M NaCl, 0.1M NaOAc, pH 4

30kDa Centriprep concentrating columns

Cyanogen bromide activated Sepharose 4B beads (Pharmacia, UK)

Erylyse Per 100ml: 90ml 0.16M NH$_4$Cl, 10ml TRIS-Cl 0.17M pH 7.65. Adjust pH to 7.2 with HCl.

TBS 25mM TRIS-base, pH 8.

Cations 1mM CaCl$_2$, 1mM MgCl$_2$, 10μM ZnCl$_2$ (final concentration in buffers)

IA lysis buffer TBS, cations, 2mM PMSF, 1% NP40

IA loading buffer TBS, cations, 2mM PMSF.
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IA washing buffer  IA loading buffer, 0.5M NaCl.

2.20.2 Method of column generation
This method was performed as described by the manufacturer (205). Briefly, 10mg of purified antibody was dialysed overnight against IA carbonate buffer at 4°C and then concentrated to >1mg/ml by centrifugation in a 30kDa Centriprep. Meanwhile, 0.5g of freeze-dried Sepharose 4B beads were resuspended in 5ml 1mM HCl and washed in 1mM HCl over 30 minutes at room temperature. The HCl was then removed with a pipette until the gel appeared dry. 5mg of antibody solution was then added to 1ml of beads and the mixture mixed gently, overnight at 4°C. Any remaining active sites were then blocked by removing the antibody solution and adding 10ml 0.2M glycine pH 8, per ml of gel. This mixture was then incubated for 2 hours at room temperature. This solution was then removed and the beads were washed in IA carbonate buffer followed by IA acetate buffer followed by IA carbonate buffer. Beads were then poured into an open ended 2ml column and allowed to settle. The column was then stored in PBS and 0.02% sodium azide at 4°C.

2.20.3 Immunoaffinity purification method
3 mice per purification were sacrificed, the hind legs removed and the bone marrow purified. This was done by removing the ends of the bones and flushing out the marrow with PBS. Cells were then pelleted (5 minutes at 284g, 1,000RPM in a JS4.2 rotor), the supernatant removed and the erythrocytes removed by the addition of 1.5ml erylyse. After 2 minutes at room temperature, an excess of PBS was added and the cells re-pelleted. Cells were then counted and an equal number, per purification, added to 1ml of IA lysis buffer in a 1.5ml Eppendorf. Cells were allowed to lyse for 30 minutes at 4°C. Membranes and nuclei were then pelleted by a 10 minute centrifugation at 9,464g (13,000RPM in a microfuge) and 4°C. The supernatant was then used to load the column.

A new column was used for each set of 2 immunoaffinity purifications. Columns were pre-equilibrated with IA loading buffer, prior to loading with the cell lysate. Columns were then washed with 5 load volumes of IA loading buffer, followed by 5 load volumes of IA washing buffer. Bound proteins were finally eluted with the
addition of 5ml 1mM HCl. All fractions were separated on a 15% SDS-PAGE gel and visualised by silver staining.

2.21 Western blotting

2.21.1 Materials

Wet blotting apparatus

PBS-Tween                PBS + 0.1% Tween-20
Blocking agent           PBS-Tween + 5% milk powder
Ponceau S stain

Nitro-cellulose membranes (Amersham)

Rabbit polyclonal antibodies  Anti MRP-8p, NH-9 test bleed I (1:100)
                               Anti-MRP-14, NH-6 (1:10,000)
Rat monoclonal antibodies    Anti MRP-8p, test sample (1:100)
                               Anti MRP-14, 2B10, tissue culture supernatant (1:25)
Transfer buffer              Per 2500ml, 250ml 250mM TRIS-base/1.92M glycine, 500ml methanol, 1750ml water.

Blotting paper (3MM)

Secondary antibodies       Goat anti-rabbit HRP conjugate (1:2000)
                           Sheep anti-rat HRP conjugate (1:2000)

Developing solution (Amersham)

2.21.2 Method

To achieve results with this method, it is necessary to complete the entire operation in 1 day. Bone marrow cell lysates were prepared as described in section 2.20.3 (page 103) except that lysates were prepared from 5 x 10^6 cells in 100μl lysis buffer, for each mouse. 30μl (1.5 x 10^6 cells) of lysate was then run in each lane of a 15% SDS-PAGE gel. Gels were transferred to nitro-cellulose membrane in a wet blotting system.

Briefly, wearing gloves, the transfer cassette and gauze were soaked in transfer buffer. The cassette was opened and upon the black side was lain 2 pieces of 3MM blotting paper, soaked in transfer buffer. On top of this was then lain the gel to be transferred, the nitrocellulose membrane, 2 more sheets of 3MM paper and the other piece of gauze. Air bubbles were removed via rolling with a pipette. The cassette was then closed and placed in the transfer apparatus, black cassette side facing the cathode.
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The apparatus was then run at 60v for 40 minutes. Transfer was checked by staining the nitrocellulose membrane with Ponceau-S for 5 minutes. A photocopy of the stained membrane was taken for future reference.

Blots were then placed in blocking buffer and incubated for 1 hour at room temperature with shaking. Blots were then transferred to plastic incubation sacs and incubated with the appropriate concentration of primary antibody for 1 hour at room temperature with agitation. Blots were then washed 3 times for 5 minutes, at room temperature, in PBS-Tween. Blots were then transferred back into sacs and incubated with the appropriate secondary antibody, for 1 hour at room temperature, with agitation. Blots were then washed 3 times for 5 minutes, at room temperature, in PBS-Tween.

Blots were developed by the addition of 3ml per 54cm$^2$ area of blot of developing solution. This was allowed to incubate for 1 minute at room temperature, after which time it was removed and the blot exposed to film.

2.22 Statistical analysis of data

Data presented in this thesis is generally presented as a mean average plus or minus the standard error of the mean (SEM). SEM is calculated from the standard deviation (SD), as follows SEM = SD/((n-1)^0.5), where n is the number of samples. Further statistical analysis of data was not performed as the sample numbers were generally too small to make any statistical conclusions drawn more meaningful than simple observation of non-overlapping means and SEMs.
3. Cloning and purification of recombinant MRP-8 and MRP-14

3.1 Introduction

As was described in the introduction, MRP-8 and MRP-14 are very highly expressed proteins which only recently have started to reveal their function. Some years ago, murine MRP-8, also known as CP-10, was found to be a highly potent chemoattractant for neutrophils. Human MRP-8 is not chemotactic.

Functional data about murine MRP-14 is sparse although MRP-14 is reported to exhibit MDF-like activity and, when coupled to beads, to recruit neutrophils over a 2 week period, in vivo. Previous work in this laboratory has shown that human MRP-14 is a novel activator of Mac-1 and moreover, that human MRP-8 negatively regulates this activation by the formation of a MRP-8/MRP-14 heterodimeric complex.

The aims of this thesis are to further investigate the function of MRP-8 and MRP-14 by the cloning, purification and biological characterisation of the recombinant proteins and also by the generation of knockout mice (described in chapters 5 and 6).

The rationale for cloning the proteins must first be addressed. It was decided to clone the murine proteins, even though the laboratory had both human MRP-8 and MRP-14 recombinant proteins and also some human functional data. This decision was taken, firstly, to allow comparison of biological function between the human and murine proteins so that the relevance of any conclusions drawn from the knockout models could be evaluated. Secondly, production of the murine proteins was undertaken to allow the generation of both polyclonal and monoclonal antibodies which would potentially be very important for both recombinant protein experiments and the future characterisation of the knockout mice.

This chapter aims to describe the cloning and purification of murine MRP-8 and MRP-14 and also to define some of the basic biochemical features of MRP-14.

3.2 cDNA cloning of MRP-8 and MRP-14

Initial attempts to clone MRP-8 and MRP-14 cDNAs centred around the use of 5' and 3' primers which both contained a Ndel site, situated 5' and 3' to the regions of coding homology, respectively. Using this NdeI site, subsequent PCR products could then be cloned into the protein expression vector pET-3a. This vector is a simple,
Chapter 3: Cloning and purification of recombinant MRP-8 and MRP-14

An early-generation, protein expression vector which contains only a C-terminal T7 tag, which is not utilised if cloning a sequence with NdeI termini and a stop codon. The cDNA termini of MRP-8 and MRP-14 do not naturally contain NdeI sites, so homologous sequence was supplied either side of the engineered site to allow priming. A schematic of the primer structure is shown in Figure 3.1 (on page 110).

PCR products were generated using these primers and a bone marrow cDNA library. The products were then gene-cleansed and digested with NdeI, to allow cloning into the NdeI-linearised pET-3a vector. The results of this strategy were disappointing. Few products were cloned and those that were cloned, upon initial analysis were the correct length for the gene in question, 270bp for MRP-8 and 368bp for MRP14. However, after sequencing, these clones were shown to be genetic artefacts, that is they did not contain MRP-8, MRP-14 or any recognisable DNA sequences, just apparently random bases with assorted stop codons.

The reasons for the initial failure to clone the cDNAs may be threefold. First, as a general rule, enzymes require 6 bases pairs on either side of their recognition site to cleave efficiently (NEB, technical information) and only 4 were provided on the 5’- (for the forward) and 3’- (for the back) in the original primer sets. Therefore, of the products that were produced, many may have been lost at the cloning step, as they may never have ended up with termini which could be ligated. This would reduce the number of clones available for analysis. Secondly, the failure may have been due to the short length of the primers, internal to the cDNA sequences (18 bases) or to a failure of the 5’- most or 3’- most regions of the primers to anneal to template because of the restriction site insertion. This would increase the chance of cloning non-specific products. Thirdly, the failure may have rested with the template. Many cDNA libraries do not contain cDNAs of less than 500bp in order to minimise the cloning of cDNA artefacts within their system. However, consultation with the makers of the library (D.Simmons, Oxford) suggested that small cDNAs should indeed be represented. Furthermore, probing of the library with those primers used to generate specific genomic probes for MRP-8 and MRP-14 (8F1/8B2 and 14F2/14B2, see section 5.2.1, page 192 and section 5.3.1, page 208) suggested that the MRP-8 and MRP-14 cDNAs were present within the library (data not shown). Taken together, these data suggested that a small change in approach was necessary to clone the MRP-8 and MRP-14 cDNAs.
The second approach used, to clone the MRP-8 and MRP-14 cDNAs, utilised the pAMP-1 vector, specialised for the cloning of PCR products. Use of this vector necessitated using primers with specialised ends (described in 2.14.2.4.1, page 82). A schematic of the primer structure is shown in Figure 3.2 (on page 110). Other primer modifications included the addition of an extra internal codon for both the forward and back primers (to increase specificity) and the relocation of the added restriction site so that it did not interrupt regions of complementarity and hence potentially interrupt annealing. In addition, a 3'-BamHI site was introduced so that cloning could be directional, (5'-Ndel, 3'-BamHI) which avoids a second screening step for sense strand orientation. New template DNA was also used, in tandem with the bone marrow cDNA library; EST clones of MRP-8 and MRP-14 (172) were identified from a web database search and sourced from IMAGE corporation, USA.

PCR products generated from the new template and also from the bone marrow cDNA library were gene cleaned, cloned directly into pAMP-1 and transformed into *E. coli* XL-1 Blue. Colonies were then screened with the internal primers 8F1 and 8F2 for MRP-8 and 14F2 and 14B2 for MRP-14. Positive colonies were then sequenced and positively identified (data not shown). MRP-14 and a mutant MRP-8 were cloned from the bone marrow cDNA library (MRP-8 had amino acid 79 mutated from F to Y, known as MRP-8FY), MRP-8 was cloned from the MRP-8 EST clone. MRP-8, MRP-8FY and MRP-14 cDNA clones were subsequently transferred into the pET-3a vector, for improved protein expression, by virtue of the 5'-Ndel and 3'-BamHI restriction sites and again transformed into *E. coli* XL1-Blue. These clones were known as 3a-8, 3a-8FY and 3a-14.

Colonies were again screened by PCR and positive clones re-sequenced (data not shown). Final positive verification of identity was followed by transformation into *E. coli* BL21 (DE3) pLysS, which is favoured for protein production rather than cloning.

The choice of protein expression bacteria was based on the presence of the DE3 plasmid in BL21 which allows IPTG induction of the T7 polymerase and also the pLysS plasmid which prevents leaky expression of T7 polymerase, and hence protects the bacteria from potentially harmful protein products.

The cDNA sequences for MRP-8, MRP-8FY and MRP-14 were later transferred into the His-tag vector, pET-28a. This was achieved simply; sequences were excised from pET-3a by Ndel/BamHI excision, and cloned into pET-28a which had
been linearised with NdeI/BamHI. Vectors were then transformed into XL-1 blue. Recombinant colonies were assessed by PCR analysis with internal primers, as described above and positive recombinants were then transformed into E.coli. BL21 (DE3) pLysS. These clones were known as 28a-8, 28a-FY and 28a-14.
Figure 3.1: Schematic representation of the primers initially used to clone MRP-8 and MRP-14 cDNAs into the pET-3a vector. Bases in curved text represent those that do not directly complement bases in the endogenous sequence. Bases circled with a line represent the NdeI restriction sites. Start and stop refer to the translational start and stop sites.

Figure 3.2: Schematic representation of the primers used to clone MRP-8 and MRP-14 cDNAs into the pAMP-1 vector. Bases in curved text represent those that do not directly complement bases in the endogenous sequence. Bases circled with a line represent the NdeI in the F primers and BamHI restriction sites in the B primers. Start and stop refer to the translational start and stop sites.
3.3 MRP-8 protein purification

The protein purification schemes for MRP-8 and MRP-14 are different and the problems faced in the purification of each molecule were distinct. Therefore, the purification of each molecule has been addressed in separate sections. This section describes the steps taken and the attempts made to purify recombinant MRP-8 protein. The pET-3a work was carried out with the intellectual input of Matthew Robinson (ICRF).

3.3.1 pET-3a

At the outset of the purification investigations, it was decided to try and pursue purification of both the wild-type and mutant MRP-8 molecules. The first step in purification was to characterise the induction parameters for the molecule. Both MRP-8 and MRP-8FY share similar parameters; maximal induction was found at 5 hours after a 0.4mM IPTG induction at 37°C. Interestingly, MRP-8FY seemed to run very differently, at 6.5kDa, on a 15% SDS-PAGE gel, compared to MRP-8, which ran at approximately 10kDa (see Figure 3.3B, on page 116). Upon cellular fractionation, MRP-8 and MRP-8FY were found mainly in the inclusion bodies and not in the supernatant (Figure 3.3B). Milder induction conditions are considered to decrease the probability of a protein being designated “toxic” and hence translocated to the inclusion bodies. Therefore, a downwards IPTG concentration titration was performed. Alteration of IPTG concentration used in the induction from 0.4mM, down to 0.1mM did not alter the protein distribution within the bacteria (data not shown).

The sub-cellular distribution characteristics of MRP-8 and MRP-8FY are very similar to those found for human MRP-8 (M. Robinson, personal communication). Therefore, the main problem with MRP-8 production would probably not be purification, as protein in the inclusion bodies can be 90% pure after an inclusion body preparation, but rather protein re-naturation. The protocol for purification of human MRP-8 is an isoelectric focusing step, followed by protein solubilisation and selection on a hydroxyapatite column. A protocol based on these steps was the one that was initially attempted to purify murine MRP-8.

A schematic summary of all the steps attempted to purify MRP-8 is detailed in Figure 3.3A. After induction, MRP-8 pellets were washed in PBS and stored at -20°C. Pellets were then thawed in buffer A with added DNase to reduce the viscosity of the
samples and allow manipulation. *E.coli* are quite fragile when frozen without glycerol and lyse upon thawing, releasing all their DNA and generating a very viscous ‘soup’. The resultant ‘soup’ was put through an inclusion body preparation and the MRP-8 poor supernatant (cytosolic fraction) discarded. The MRP-8 rich, inclusion body pellet was solubilised in 6M urea, overnight, at room temperature with gentle agitation.

The MRP-8 was then subjected to isoelectric focusing (IEF) over a pH 5-7 gradient (computer predicted IEP was 5.55); this did not cause sharp focusing but rather a blurred focusing and substantial precipitation of material (Figure 3.3C); this is a common feature of the human purification protocol (M. Robinson, personal communication). However, the focusing achieved with the pH 5-7 gradient was superior to a 3-10 gradient (data not shown). This focused material was again resolubilised, overnight, in its own buffer. This step provided only a marginal increase in purity (see Figure 3.3C, compare with ‘L’ in Figure 3.3C).

As the IEF produced only a marginal increase in protein purity, from the 95% purity of the inclusion body preparation, attempts were made to resolubilise the MRP-8 by simple dialysis out of urea and hence away from denaturation agents, without the IEF (see Figure 3.3A for explanation of event sequence). Whether this was attempted stepwise with 6M protein into 5M, 4M, 3M etc. levels of urea or rapidly, into 0M urea, the end result was identical; complete precipitation of the protein with none left in solution (as evidenced by protein estimation and also as visualised by SDS-PAGE). The precipitated material could not be resolubilised into urea.

After the IEF step, investigations into resolubilisation branched out. As had been attempted with the non-IEF treated MRP-8, either a stepwise or rapid protocol was followed. Both protocols resulted in a significant amount of protein precipitation but significantly, there appeared to be soluble material remaining (by protein estimation). Therefore, the final step in the human protocol, purification on hydroxyapatite was attempted.

Hydroxyapatite binds only calcium binding proteins. Therefore, it was ideal for the purification of EF-hand containing proteins such as MRP-8 and MRP-14. This selection step had a second level of complexity in that it would clearly only bind functional calcium binding proteins, that is, correctly folded proteins. Therefore, a protein that had not folded correctly would not bind to the column and would flow through to waste. This assumption produced an observation about the refolding.
protocols that had been attempted; the rapid renaturation protocol produced an approximately double flow through of material compared to the slow renaturation protocol, for a given amount of starting material (data not shown). This suggested that the rapid renaturation protocol produced a higher percentage of incorrectly folded material than did the slow renaturation protocol. The end result of the hydroxyapatite purification of MRP-8 from either renaturation protocol was a peak visible to the spectrophotometer at 25-30%B and 280nm. However, this material was not visible on SDS-PAGE gels stained with coomassie or silver stain. Protein estimation suggested the presence of very small amounts of material (200μg/0.5L with slow renaturation and 80μg/0.5L with fast renaturation). Whether this was real or an artefact of the protein estimation system, given that the levels were so low, remains to be seen. It is thought that the difference between the initial spectrophotometer readings and the later gel analysis results were due to precipitation rather than a buffer effect as HA-B buffer does not absorb at 280nm. Whatever the answer was, this method was not going to produce material stable enough to work with, or enough material for functional assays or immunisations.

Given that some soluble MRP-8 was produced in the E.coli and that the hydroxyapatite column system was able to select for calcium-binding proteins, an attempt was made to purify soluble MRP-8, directly from the bacterial cytosol, thereby obviating any resolubilisation problems. The original hypothesis was that only MRP-8 would be bound to the column. Therefore, in theory a large amount of bacterial product could be added to the column to make up for the low cytosolic expression. A cytosolic preparation was made from an induced bacterial pellet and this was initially purified by centrifugation and 0.45μm filtration. After loading onto a hydroxyapatite column was performed, soluble MRP-8 was retrieved, eluting at 25%B (125mM phosphate), but it was approximately 10% pure, (see Figure 3.3D, lanes marked ‘*’, compare with ‘8 SN’ in Figure 3.3B). Moreover, the protocol was clumsy as there was a very long wash period to remove non-specifically associated bacterial proteins from the column. Therefore, it was decided that this protocol was also not viable for the production of MRP-8.

No mention has been made in this description of the attempted purification of MRP-8, about the MRP-8 mutant, MRP-8FY. Purification attempts were not made for this molecule because of the failure of the purification of the wild type molecule.
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The one hopeful aspect about the purification of MRP-8 was the seeming fluid phase stability of the molecule, that is to say, the ability to relatively purify the molecule from the bacterial cytosol without precipitation. This observation produced that idea that the way forward would be to obviate the negative features of hydroxyapatite purification of bacterial cytosol i.e. the contamination. The best way to do this was to attempt purification of MRP-8, after affinity tagging.

3.3.2 pET-28a

After the relative failure of either MRP-8 or MRP-14 (see section 3.4.1) purification using an untagged recombinant protein, the decision was taken to tag the molecules for purification. This was not initially attempted as proteins cloned in tagged vectors are either permanently fused to the tags or, after tag removal, a number of additional amino acids remain that could potentially alter protein function. However, molecule tagging seemed to be the only way forward and so the molecules were conveniently cloned into pET-28a, a His tagged vector. This vector allows either N- or C- terminal his-tagging with 6 consecutive His residues. Both molecules were N-terminally tagged because (a) it was easier as the sites were exactly the same in pET-3a and (b) this allowed tag removal by thrombin, although this leaves the following 3 amino acids, before the methionine of the protein of interest, Gly-Ser-His. C-terminal tagging was not considered as it had various disadvantages, including an inability to remove the His-tag after protein production.

The initial step in the purification of His-MRP-8 was to evaluate the subcellular localisation; this was found to be split evenly between the inclusion bodies and the cytosol (see Figure 3.4B, on page 117). The optimal conditions for cytosolic expression of MRP-8 appeared to be 0.4mM IPTG for 5 hours at 37°C; optimal MRP-8FY expression was identical except that the optimal incubation temperature was 28°C. Increasing IPTG concentrations up to 1mM did not increase the amount of induction, as visualised by SDS-PAGE (data not shown).

Tagging MRP-8 had a profound effect on the calculated IEP, moving it from 5.55 to 6.8, perhaps this change is associated with the relative increase in protein solubility (compare to untagged MRP-8 in Figure 3.3B). Interestingly, MRP-8 was still substantially larger than MRP-8FY; both proteins changed apparent molecular mass by
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SDS-PAGE (MRP-8 from 10kDa to 19kDa, and MRP-8FY from 6kDa to 10.5kDa) (see Figure 3.4B, on page 117).

Purification of His-MRP-8 from bacterial supernatant was relatively straightforward. An overview of the steps taken is described in Figure 3.4A. Although the protein could be purified to homogeneity (Figure 3.4C) eluting at 27-30%B, the yield of protein, at 0.5mg/L (starting culture) was low. Furthermore, this protein precipitated within 6 days, when stored at 4°C and overnight if left at room temperature.

Attempts to cleave the tag from the protein resulted in complete precipitation of the protein, if the cleavage was performed at 37°C and approximately 50% if performed at room temperature. As the yield prior to this step was 0.5mg/L, progression towards cleaved, purified protein, dialysed into an assay buffer that would not affect cells was increasingly difficult. Attempts to dialyse the His-tagged protein from the final imidazole and salt rich buffer were also relatively unsuccessful; as evident from precipitation and a recovery of approximately 100µg from a 1mg pre-dialysis starting point, followed by further protein precipitation over 48h. At this point, the attempts to generate murine MRP-8 in order to confirm the results of the Geczy group (137) were abandoned. Protein was henceforth routinely generated as a His-tagged product in order to immunise rabbits, every time an immunogenic boost was required.
Figure 3.3: Schematic diagram characterising the attempts made to purify untagged rMRP-8.
A. Overall schematic of the steps taken. Bold letters cross-reference other sections of the figure. B. Cellular sublocalisation of MRP-8 and MRP-8FY, time of induction in hours is shown above each species; P and SN refer to pellet and supernatant, respectively. C. Isoelectric focusing of MRP-8, derived from inclusion bodies, over a pH range from 5 to 7; L denotes sample prior to focusing. D. Hydroxyapatite purification of MRP-8 from a supernatant preparation. * refers to samples containing MRP-8.
Figure 3.4: Schematic diagram characterising the attempts made to purify tagged rMRP-8.

A. Overall schematic of the steps taken. Bold letters cross-reference other sections of the figure.

B. Cellular sublocalisation of MRP-8 and MRP-8FY, time of induction in hours is shown above each species; P and SN refer to pellet and supernatant, respectively; M refers to molecular weight markers.

C. Nickel affinity purification of MRP-8 from a supernatant preparation; L refers to load.
3.4 MRP-14 protein purification

This section describes the steps taken and the attempts made to purify recombinant MRP-14 protein. The pET-3a work was carried out with the intellectual input of Matthew Robinson.

3.4.1 pET-3a

The purification protocol designed and tested for MRP-14 is distinct from that used to purify MRP-8 mainly because MRP-14 is soluble. Sublocalisation investigations revealed that MRP-14 was found almost exclusively in the cytoplasm (Figure 3.5B, page 122). That is to say, MRP-14 is not aggregated and denatured by translocation to inclusion bodies after synthesis but remains in the cytosol. The initial sublocalisation data provided a hope for eventual purification to homogeneity as purification without a refolding step is acknowledged to be simpler than purification incorporating refolding steps.

Initial efforts to purify MRP-14 were based around the protocol used to purify human MRP-14 (although human MRP-14 is found in the inclusion bodies) as both MRP-14 molecules are obviously homologous and may share various biochemical characteristics allowing analogous purification. The purification of human MRP-14 involved separation on a Mono P chromatofocusing column, followed by selection on a hydroxyapatite column.

The starting point for all attempts to purify MRP-14 was a cytosolic protein preparation. A schematic summary of all the steps attempted is detailed in Figure 3.5A (page 122). The first step to be attempted was a Mono-P chromatofocusing step, this enriched MRP-14 protein purity from approximately 10% to 60% purity (Figure 3.5F, impurities are marked with arrows). This 60%-pure MRP-14 was then subjected to hydroxyapatite selection. Peak protein elution was found at 15%B (75mM phosphate). This step resulted in an enrichment of MRP-14 protein purity to approximately 95%. Unfortunately, homogeneity, as assessed by coomassie stain of an SDS-PAGE gel could not be achieved (data not shown).

One obvious problem area was the initial Mono P focusing step. Mono P columns are focusing, similar to the IEF apparatus in mechanism, rather than selection columns, so that all the material loaded onto the column elutes from the column once the pH gradient is initiated. This step is fine for relatively pure material, such as that
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purified from inclusion bodies (as is the case for human MRP-14), but it is less efficient for cytosolic protein separation where many protein species are loaded onto the column. Another disadvantage was the number of Mono P runs required to produce a good batch of material. For example, of 20mg protein loaded, 6mg of 60% pure MRP-14 was recovered. The other area of concern was the failure to reach homogeneity with hydroxyapatite selection, but this was to be addressed later.

The modified purification protocol involved performing a selection step first, in order to remove most of the unwanted material. Therefore, cytosolic protein was loaded onto hydroxyapatite and removed in a stepwise fashion. A large portion of unwanted protein was removed with an initial, low stringency 0.5%B wash; MRP-14 eluted at approximately 5%B although it co-eluted with a number of contaminating bands (approximately 75% pure) (Figure 3.5C, impurities marked with arrows). At this stage, there was 45mg of 75% pure MRP-14 from 0.3L of starting culture pellet.

The 75% pure MRP-14 was then chromatofocused on a Mono P column. The resultant material was approximately 95% pure (Figure 3.5D, impurities marked with arrows), therefore a final hydroxyapatite selection run was performed. A substantial amount of material was lost at the Mono P chromatofocusing stage, at least partially due to Mono P column flow through.

The final hydroxyapatite separation resulted in approximately 15mg of 98% pure material by coomassie stain (Figure 3.5E, impurities marked with arrows). However, it was decided that this was not ideal for the in vivo applications that were planned and so, in common with MRP-8, an attempt was made to tag the protein for purification to homogeneity.

3.4.2 pET-28a

Initial induction profiling and sublocalisation studies revealed that His-MRP-14 was very similar in profile to non-tagged MRP-14. Protein was predominantly located within the soluble cytosolic fraction (Figure 3.6B, on page 122) and the best induction conditions were found to be a 5 hour incubation with 0.7mM IPTG at 37°C. Tagging of MRP-14 caused a predicted IEP shift from 7.17 to 7.73 and a molecular weight shift from 14 to approximately 16kDa.

As was found with His-MRP-8, purification of His-MRP-14 was simple. An overview of the steps taken is described in Figure 3.6A (page 123). Protein was purified
to homogeneity after the His-column selection step, although a substantial portion was found as a dimer (Figure 3.6C). The yield of His-MRP-14 was excellent; purified protein amounts ranged from 30-60mg/L of original induced culture.

Although pure protein was available at this stage, it was thought that biological assays would have less possible artefacts and also would not need a tagged control protein if the tag was removed. To this end, a titration of thrombin concentrations, incubation times and temperatures was performed; this suggested that optimal cleavage conditions were 1U/100μg, digested overnight (18h maximum) at room temperature (as recommended by Pharmacia). Use of this set of digestion conditions seemed to result in complete digestion of the tagged protein. This was evidenced on SDS-PAGE as a shift in molecular mass from 18kDa to 14kDa (compare Figure 3.6C, “Pooled fractions” with Figure 3.6D). Interestingly, neither the tag (approximately 2kDa) nor the enzyme (36kDa) could be seen.

The final stage in the purification of MRP-14 was to purify the MRP-14 protein away from the His-tag and the thrombin protease. An ideal method of doing this seemed to be via hydroxyapatite selection. In theory, the his-tag and thrombin protease should flow through the column whereas the MRP-14 should concentrate on the matrix. MRP-14 had left the nickel affinity column in a 500mM NaCl and approximately 300mM imidazole buffer, and then digested in the same buffer. Therefore, initial efforts were made to dialyse the MRP-14 into HA-A, in order to maximise the efficiency of the purification. As imidazole interferes with nickel binding, it would probably interfere with calcium binding also. Unfortunately, this particular step resulted in significant protein precipitation, and therefore another approach was sought.

A solution to the problem was reached; the imidazole-rich protein solution was diluted 1:5 with water to lower the imidazole concentration to approximately 60mM and the NaCl to 100mM. 1:100 of HA-B buffer was added to the mixture to complete the approximation of the new buffer to the hydroxyapatite loading buffer, HA-A. The imidazole concentration was significantly higher than the loading concentration of 5mM, found in the nickel affinity protocol. However, MRP-14 did bind to the hydroxyapatite at this concentration and there was a fringe benefit of a strong selective pressure against the binding of other proteins.

MRP-14 was successfully purified to homogeneity using this method (Figure 3.6D). Although there was a flow-through as measured by absorbance at OD$_{280}$, this
was thought to be a combination of imidazole (which absorbs strongly at OD$_{280}$) and the flow through of thrombin and His-tag; no MRP-14 was visible in the flow through (data not shown). Finally, pure MRP-14 was dialysed overnight at 4°C, into Hanks-HEPES, filtered for sterility and stored at -70°C. The final yield varied from 20 to 40mg/L.
Figure 3.5: Schematic diagram characterising the attempts made to purify untagged rMRP-14.

A. Overall schematic of the steps taken. Bold letters cross reference other sections of the figure. B. Cellular sublocalisation of MRP-14 after a 3h induction; P and SN refer to pellet and supernatant, respectively. C. Hydroxyapatite purification of MRP-14 from a supernatant preparation; arrows highlight impurities. D. Mono P chromatofocusing of hydroxyapatite purified MRP-14, over a pH range from 3 to 7. E. Final hydroxyapatite purification of MRP-14 that has been purified via C. and D. F. Mono P chromatofocusing of MRP-14 from a supernatant preparation, over a pH range from 3 to 7.
Figure 3.6: Schematic diagram characterising the attempts made to purify tagged rMRP-14. A. Overall schematic of the steps taken. Bold letters cross reference other sections of the figure. B. Cellular sublocalisation of MRP-14, induction time and temperature are shown; P and SN refer to pellet and supernatant, respectively; M refers to molecular weight markers. C. Nickel affinity purification of His-MRP-14 from a supernatant preparation; L refers to load. D. Hydroxyapatite purification of MRP-14 from C.
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3.5 Biochemical analysis of MRP-14

Once MRP-14 had been purified to homogeneity, the first questions to be addressed were biochemical in nature. Is the protein that has been produced really MRP-14? Has the purification process resulted in the generation of aggregated protein that may not be functional in biological assays? Does the protein solution that has been generated contain only MRP-14 or are there other contaminants? To answer these questions, a few basic analyses were carried out, as detailed below.

3.5.1 MALDI

The identity of MRP-14 was further confirmed using matrix assisted laser desorption mass spectrometry analysis. This consists of digesting the protein of interest with various enzymes and analysing the molecular mass of the generated fragments with a mass spectrometer. From this, a prediction of the identity of the original protein can be made. Obviously, if the identity is already known, a confirmation of identity can be performed. This work and its subsequent analysis was performed in the laboratory of Darryl Pappin (ICRF, UK).

Tryptic digestion of MRP-14 produced two diagnostic peaks, with a molecular mass of 1682.9, corresponding to SITIITIDTFHQYSR, and 1429.7, corresponding to QMVEAQLATFMK. V8 digestion produced the diagnostic peak of 2096.352, corresponding to RSITIITIDTFHQYSRKE. Together, these data suggest that the MRP-14 generated from the pET-28a vector had the same sequence as the published protein (140) and therefore was the same molecule.

3.5.2 Laser light scattering

This work was carried out by Arnold Coffer (ICRF, UK). The average molecular weight of a protein sample can be estimated using the laser light scattering technique. This information can provide an indication about the aggregation state of the sample. For example, if the average molecular weight of a 10kDa protein was found to be 30kDa, this would suggest the protein was predominantly existing in solution as a trimer. When MRP-14 was analysed in this fashion, it was found to have a molecular mass of 28±2 kDa, suggesting that the protein was predominantly found in solution as a dimer.
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3.5.3 Thrombin contamination

The method used to remove the His-tag from MRP-14 was protease digestion mediated by bovine thrombin. Thrombin is well known in biology to have a plethora of effects and therefore its presence in a sample of recombinant protein whose function is under investigation would be highly undesirable. Throughout the course of most of the investigations described in chapter 4, it had been assumed that the added thrombin was lost during the hydroxyapatite selection process, as it is not a calcium binding protein like MRP-14. As no contaminating bands were present at 37kDa, the molecular weight of thrombin, during purification of any of the batches of MRP-14, further investigation of potential thrombin contamination was not pursued.

Later results (described in Chapter 4), led this investigator to test the hypothesis that there could be a carry-through of thrombin in the purification which was generating the biological phenomenon observed. To assay for thrombin contamination within a preparation of MRP-14, use was made of the chromogenic substrate S2238. S2238 is a peptidic substrate which when cleaved by thrombin releases a pNA group which is fluorescent at 405nm. Unfortunately, the contamination hypothesis was correct and approximately 60% of the added amount of thrombin co-purified with MRP-14. This contaminating level approximated to 2 NIH U/ml and the protein concentrations varied from 400µg/ml to 800 µg/ml. This contamination of 2NIH U/ml approximated to 1.32µg/ml of thrombin protein, which was why it was not visible on an SDS-PAGE gel.

The next question was, how could the contamination be removed? Initial investigations with agarose-bead-coupled p-aminobenzamidine (105), were a failure, resulting in no decrease in the chromogenic thrombin activity of the contaminated samples. The next method to be tried was the use of the endogenous regulatory factor, anti-thrombin III, again coupled to agarose beads. This approach was much more successful, resulting in complete removal of thrombin, as assayed by chromogenic activity. This step was inserted into the MRP-14 purification scheme, prior to the final hydroxyapatite purification.
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3.6 Discussion

3.6.1 MRP-8

One interesting feature of the cloning of MRP-8 was the generation of the mutant molecule, 8FY, from a bone marrow cDNA library. The FY mutation is a phenyl alanine to tyrosine mutation in the second EF hand. Specifically, this mutation is the first residue in the calcium binding loop after the α-helix. Initial attempts to purify 8FY, synthesised from the pET-3a vector were a failure although basic SDS-PAGE analysis revealed that the mutation caused a large difference in size, versus the wild-type protein (6.5kDa versus 10kDa). If a purification scheme was developed to produce pure MRP-8 it would be very interesting to concomitantly purify 8FY to assess its calcium binding properties, along with all the other functions reported for MRP-8, such as in vitro and in vivo chemotaxis (137, 138).

A secondary observation related to the relative sizes of MRP-8 and 8FY was the relative change in molecular mass, as assessed by SDS-PAGE when the molecules were his-tagged. The his-tag should have a molecular mass of approximately 2kDa and protrude from the rest of the recombinant protein so that it can be selected for on nickel affinity columns. His tagging of MRP-8 increased the apparent mass from 10kDa to 20kDa, tagging 8FY increased the mass from 6kDa to 10kDa whereas tagging of MRP-14 increased the MRP-14 mass from 14kDa to 16kDa. It remains a possibility that the his-tag attached to the N-terminal of MRP-8 and 8FY was folded into the 3 dimensional structure of the recombinant protein and significantly altered it, thereby retarding their migration on a SDS-PAGE gel. If this supposition is true, then it would be of benefit to re-attempt purification of MRP-8 and 8FY from the untagged pET-3a vector.

The chief problem encountered when attempting to generate relevant amounts of functional protein centred on the relative stability of MRP-8. The main problem with untagged MRP-8 production was resolubilising protein, back to a folded form. When purifying from soluble material, the main problems were rapid precipitation from solution and also the small amount of protein generated.

When comparing the amounts of protein generated from the tagged and untagged vectors, for both MRP-8 and MRP-14, it seemed immediately obvious that more untagged than tagged protein was produced. Compare, for MRP-8, Figure 3.3B and Figure 3.4B and for MRP-14 Figure 3.5B and Figure 3.6B; for both proteins, induced levels of tagged proteins appear to be approximately 50% of untagged levels. The final,
purified amounts of tagged material is particularly important for MRP-8, given its instability.

One of the other problems in producing biologically relevant MRP-8 from the pET-28a vector was the low, final concentration after purification; peak values were 100µg/ml (total yield 800µg from a lysate derived from 2L induced bacteria). This meant that dialysis was difficult as volume expansion nearly always occurs, at least 1mg protein sticks to the amount of tubing used for 25ml product and the volume to be dialysed against needs to be 100 to 1000 fold that of the protein of interest. This all points back to needing to increase the final preparative concentration and amount of protein. In theory, one way to address this would be to increase the amount of protein attached to column prior to elution. Again, in theory, it is possible to run the lysate from 20L down a nickel affinity column and only retain the product of interest. In practice, contaminants saturate the column when using lysates from original volumes of greater than 2L.

The other problem encountered when trying to purify MRP-8 from tagged material was tag removal. It has been reported, using a GST-MRP-8 fusion protein, that facile tag removal could be achieved using thrombin at an incubation temperature of 37°C (113). Attempts to translate these results into the His-tagged system resulted in complete precipitation of the protein, if the cleavage was performed at 37°C and approximately 50% if performed at room temperature. However, this observation is not really relevant given the general stability of the protein and the amounts generated.

A future investigator may wish to reproduce previously published work exactly; that is, to generate GST-MRP-8 as has been described (113). In that report, cleaved protein was produced at approximately 5mg/L starting culture which would be enough to attempt the reproduction of the published functional data (50), data which has yet to be reproduced in another laboratory. The only problems with this approach may be the sourcing and use of 2 reverse-phase HPLC columns, C₈ and C₁₈, respectively. The decision not to attempt GST-tagging but to use His-tagging of MRP-8 and MRP-14 was taken with knowledge of the reported success of the GST approach (for MRP-8) (113) and was based on the following information. The vectors for His-tagging had the same restriction sites as the untagged vectors, allowing facile cDNA transfer. In contrast, the GST-tagging vectors investigated had sites that would have required cDNA modification after excision from the untagged vector.
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Given the difficulties encountered when producing MRP-8 from the soluble fraction of a tagged bacterial lysate and the potential difficulties with reproducing the published GST-purification/thrombin cleavage data, it may be worth attempting to optimise the resolubilisation of non-tagged MRP-8. The purity of the inclusion-body bound material was approximately 90% before any manipulations and the yield was potentially >60mg/L. Therefore, if there is a way to resolubilise this material, followed by a folding selective step, such as hydroxyapatite, this would be an ideal method to produce protein.

3.6.2 MRP-14

Initial attempts to purify MRP-14, using untagged protein were partially successful. After a hydroxyapatite selection, a Mono P chromatofocusing and a final hydroxyapatite separation, the final purified MRP-14 was approximately 98% pure, as judged by a coomassie stained protein gel (Figure 3.6E, impurities marked with arrows). Unfortunately, there were at least 2 contaminating species that were not lost during purification and therefore the untagged system was not pursued.

Working with the tagged system initially seemed to be highly successful. The first step, nickel-affinity selection, was highly specific and generally purified protein to homogeneity. Thrombin-digestion mediated tag removal followed by hydroxyapatite selection also seemed successful. Indeed, the protein produced from this step was pure, by Coomassie staining (Figure 3.6D).

Further biochemical investigations such as MALDI analysis, confirmed the identity of the recombinant protein that had been purified as MRP-14. This data was supported by laser light scattering investigations.

MRP-14 was generally found as a dimer (approximately 70%). Evidence for this comes from non-reducing gels (data not shown) and also from laser light scattering (aggregometry) studies which analyse the average molecular mass of a given sample. From this, and the knowledge of the original mass of the material, a guess about the multimeric state can be made (A. Coffer, personal communication). This can also be seen by examination of the various purification stages of both the tagged and untagged material, where dimer is clearly visible, presumably because there was not enough β-mercaptoethanol present (Figure 3.5C, Figure 3.5D, Figure 3.6B). The dimer band was initially thought to be a contaminating band, until further gels with levels of β-
mercaptoethanol up to 5%, caused the band to disappear, in association with an increase in the intensity of the monomer band. Interestingly, a recent report suggests that recombinant MRP-14 exists 95% as a covalently linked dimer (209). In contrast, recombinant MRP-8, which is also chemotactic (but $10^{-11}$M versus $10^{-13}$ for native protein) (113), exists in solution, predominantly as a non-covalently linked dimer (210).

The main problem with the purification of MRP-14 from tagged material was not discovered until the end of the experimental phase of this thesis. It had been assumed that the thrombin added to the His-MRP-14 in order to cleave it to MRP-14 would be lost during the hydroxyapatite selection process. Furthermore, if any thrombin did carry through the purification process, it was thought that it would undergo autolysis and lose its biological effects. Unfortunately, this was not the case and approximately 60% of the added amount of thrombin was co-purified with the MRP-14 and the active site was functional (the active site is required for hydrolysis of the S2238 chromogenic substrate).

Investigations into removing the thrombin contamination revealed that a simple method for removal was covalent binding by the endogenous regulatory factor, antithrombin III. Using this method, prior to hydroxyapatite selection, pure non-thrombin laden-MRP-14 was finally produced. Fortunately, this thrombin-free material appeared to behave very similarly to the contaminated samples in biological systems, suggesting that the results in those systems were due to MRP-14 and not thrombin (see chapter 4).

In summary, this chapter has described the cloning of the cDNAs for MRP-8 and MRP-14. It has also described the attempted purification of untagged MRP-8 and MRP-14 recombinant protein from IPTG-induced E.coli. The chapter has also described the successful purification of MRP-8 as a his tagged protein and MRP-14 as a his tag-cleaved protein, from IPTG-induced E.coli. Finally, this chapter has also described some basic biochemical parameters for recombinant MRP-14.
4. In vitro and in vivo functional analysis of MRP-14

4.1 Introduction

This chapter addresses one of the main aims of this thesis, which is the functional characterisation of MRP-14 in vitro and in vivo. An additional purpose is to compare the functions of murine MRP-14 (muMRP-14) to human MRP-14 (huMRP-14). The reader should note that in this work MRP-14 refers to muMRP-14 unless the human protein is being concurrently mentioned in the paragraph; human MRP-14 will always be referred to as huMRP-14. The reader should also note that all mice used in this study were cared for at Lincolns Inn by Gill Hutchison and Julie Bee.

Previous investigations of huMRP-14 have revealed an interesting role in cell adhesion without cellular activation (198). The ideal next step would be to investigate the role of huMRP-14 in vivo; this is not possible for ethical reasons. However, muMRP-14 can be investigated in vivo. If muMRP-14 is found to share similar in vitro characteristics with huMRP-14, then the results of the MRP-14 in vivo investigations will allow speculation as to possible functions for huMRP-14, in vivo.

The second purpose of this in vitro and in vivo profiling is to identify further areas of potential interest in the MRP-14 knockout mice, so that these might be studied initially.

The areas that this study of MRP-14 covers include the effect of MRP-14 in vitro, on the function of various cell types including peripheral blood in assays such as adhesion, chemotaxis and cellular activation. This study also investigates MRP-14 function, in vivo, using a mouse air pouch inflammation model, a situation that cannot be ethically investigated in humans. From all this data, a potential role for MRP-14 in inflammation and leukocyte trafficking is presented.

4.2 Protein preparation - endotoxin depletion

It is well recognised that one of the major problems with recombinant protein produced in E.coli is that of endotoxin contamination. Endotoxin is a polysaccharide produced by Gram negative bacteria which coats the recombinant protein and co-purifies with the recombinant protein. Endotoxin has many and various biological effects, both pro- and anti-inflammatory, dependent on the host molecules that interact with it, for example, CD14 (273), LBP (lipopolysaccharide binding protein) (258) and BPI
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(bactericidal/permeability increasing protein) (269). Mice are reported to be substantially less sensitive to the pro-inflammatory effects of endotoxin than humans (268). However, endotoxin does mediate, the sometimes fatal septic shock and does cause release of various cytokines and the general activation of the innate immune system. Therefore, the endotoxin depletion of a recombinant protein, especially one that is to be used in the investigation of immunological functions seemed wise.

As described in the methods, all recombinant proteins and buffer controls (controls for stimulants, not all buffer used in the assay systems) were endotoxin depleted prior to usage in any of the in vitro or in vivo investigations described, by use of a detergent extraction system. This method was found to decrease the endotoxin contamination of the recombinant proteins (huMRP-14, huMRP-6, muMRP-14) by >1,000 fold (M.Robinson, personal communication). In the case of MRP-14 (at a test concentration of 540µg/ml), endotoxin levels were between 0.16µg/ml and 0.75µg/ml before endotoxin depletion and 0.163ng/ml after depletion (data not shown). This method was also associated, in many cases, with an increase in the protein concentration of the endotoxin-depleted recombinant protein (data not shown). To monitor and allow compensation for this phenomenon, protein solutions were concentration assayed prior to and following treatment.

4.3 In vitro investigations

4.3.1 Definition of the cell types used in in vitro investigations of MRP-14 function

During the course of the in vitro investigations of MRP-14 function, a number of different cell types were utilised. This section describes the development of a purification protocol for mouse neutrophils (PMN), derived from whole blood and also the composition of leukocytes found in the inflammatory infiltrate of thioglycollate-induced peritonitis. These two peripheral cell preparations were then used in the majority of the subsequent functional investigations of MRP-14. Use was also made of WEHI 265.1 cells, a monocytoid cell line derived from an Abelson virus-derived tumour in a BALB/c mouse. This cell line has various characteristics of monocytes, such as the possession of Fc receptors and the ability to phagocytose (265).
4.3.1.1 Separation of neutrophils and peripheral blood mononuclear cells from peripheral blood

After the use of WEHI 265.1 cells in the adhesion assays, it became important to know what the response of peripheral neutrophils might be to MRP-14. At this time there was not a protocol for the subfractionation of murine leukocytes so effort was invested in developing a procedure for neutrophil isolation, based on the highly successful method used to purify human neutrophils from EDTA treated blood.

In the human protocol, peripheral blood was collected in a syringe containing EDTA (to prevent clotting by chelation of calcium ions) and transferred to 15ml Falcon tubes. Dextran was then added to a final concentration of 0.6% (to precipitate the erythrocytes [RBC]) and left to sediment for 40 minutes. Percoll (density = 1.125g/ml at 100%) was diluted in PBS to 70% and 80%; the 70% layer was then layered onto the 80% layer to form a gradient for centrifugation. Densities of the various layers could be calculated as follows [density of z% Percoll = (1.125z)+((100-z)*1)]. When the dextran treated blood had sedimented, the top, straw coloured layer, containing all the leukocytes was layered on top of the 70% layer of the gradient. Cells were then separated by centrifugation for 15 minutes at 2600g. Any remaining RBC sedimented to the bottom of the vessel; neutrophils could be found at the 70% : 80% interface and the lymphocytes, monocytes and other leukocytes, plus the platelets could be found at the interface of the plasma : 70% layers. The various cell types were then harvested with plastic Pasteur pipettes, washed out of Percoll and into assay buffer.

When this method was used with murine cells, it was slightly altered. Peripheral blood was collected into PBS/EDTA, as with the human protocol, but it was then diluted 1 : 1.5 in PBS prior to dextran sedimentation. It was found that not all the RBCs pelleted in the dextran sedimentation step. This situation was only improved to 90% sedimentation by increasing the length of the incubation to 90 minutes. In the Percoll layer, density centrifugation step, cells tended to be found approximately 1 layer above the layer they were expected in; that is to say, RBC contaminated the 70% : 80% interface that contained the PMN layer and the PMN layer spread throughout the 70% layer as well as at the 70% : 80% boundary, et cetera. This situation was not improved with increasing the length of the centrifugation step by a further 20 minutes.

The first step taken in the optimisation of the protocol was to decrease the density of the Percoll bottom layer to allow the precipitation of RBC, but not
A titration showed a bottom layer of 74% to be optimal. A similar exercise with the top layer, to include the neutrophils revealed the optimal Percoll density was 64%. However, there appeared to be a 20% RBC and 5% CD3 positive contamination of the neutrophil rich band, found at the 64% : 74% interface. The most obvious improvement was to decrease the RBC contamination. This was successfully achieved by altering the dextran sedimentation step. Rather than use of 0.6% final volume dextran added to whole blood, 1.2% final volume dextran plus a 1 : 1 dilution of blood in PBS prior to sedimentation was used. The optimal sedimentation time was found to be 40 minutes. The timing of the sedimentation was important as the number of recovered neutrophils decreased although the relative lack of RBC contamination did not, if the incubation was extended.

When this altered dextran sedimentation method was included with the 64% : 74% centrifugation step, the resultant cell separation appeared to be optimal. The cell composition was 74% neutrophils (mAb 7/4), 4% T cells (mAb CD3) and 22% others, which appeared from the scatter plot to be dead cells (see Figure 4.1A, on page 135). Examination of the topmost layer, that is the plasma : 64% layer, revealed the population to be 7% neutrophils and 46% T cells, confirming that the majority of neutrophils had been present at the 64% : 74% interface (Figure 4.1A, page 135). These figures compare with erythrocyte depleted blood, which was composed of 46% PMN, 20% T-cells, 14% B-cells and 17% monocytes (see Figure 4.1B, data obtained by P. Tessier).

One major problem with this purification system was the small numbers of purified neutrophils recovered from each mouse used. Each mouse would yield approximately 1ml blood and this blood would then yield approximately 400,000 neutrophils. However, this method was henceforth used to purify neutrophils for subsequent assays.

4.3.1.2 Characterisation of leukocytes derived from thioglycollate induced peritonitis at 4 hours

As stated above, it became important to know what the response of primary, peripheral blood cells as well as cell lines would be to MRP-14. This is the reason that the protocol for isolating mouse neutrophils was developed. However, the major problem with the isolation of mouse neutrophils was the quantity of cells that could be isolated.
in practical terms. This is put into perspective when comparing the optimal number, 100,000, of human neutrophils required for 1 well of a 96-well plate adhesion assay. For the simple reason of animal usage, it was important to discover an alternative source of murine myeloid cells that could be used in the various assay systems designed to elucidate the potential functions of MRP-14.

One potential source was the thioglycollate-elicited peritonitis method which consisted of inducing a peritonitis in the mouse by the injection of thioglycollate medium. This medium is a mild irritant which contains various factors including yeast extract, agar and sodium thioglycollate. The cells elicited into the peritoneum at various time points could then be harvested. The major advantage of this method was the number and type of cells that could be recruited. For example, at the 4 hours timepoint which was used to derive cells for use in the MRP-14 in vitro analyses, the majority of cells recruited were neutrophils. Indeed, up to $10 \times 10^6$ cells per animal were recovered. Obviously, this method was particularly attractive as it decreases, drastically, the number of mice needed to procure enough cells for use in various assay systems.

When the 4 hour lavage was analysed by FACS, the cellular composition was found to be 62% PMN, 12% macrophages / monocytes, 10% B-cells and 7% T-cells. This compares quite favourably with the peripheral separation described in section 4.3.1.1. Unfortunately, this investigator was unable to further purify the elicited neutrophils using the peripheral leukocyte Percoll separation gradient, nor able to optimise the gradient for the lavage cells.
A.

Surface of 64% layer

64% : 74% interface

B.

Figure 4.1 Characterisation of the cells used in *in vitro* investigations of MRP-14 function. **A.** FACS analysis of peripheral cells separated with 64% : 74% Percoll gradients. Percentages are shown in each histogram, in brackets. **B.** Graph showing the relative leukocyte composition of whole blood (RBC sedimented), separated blood (neutrophil fraction of Percoll gradient) and thioglycollate-elicited leukocytes. Data for **B.** was obtained by P.Tessier.
4.3.2 Static adhesion assays

The first set of in vitro investigations undertaken to characterise the function of MRP-14 were static adhesion assays. As has been previously mentioned, huMRP-14 is a potent stimulator of neutrophil adhesion (198). Initial adhesion assays with human neutrophils binding human fibrinogen used huMRP-14 as well as muMRP-14. These experiments were a good system for familiarisation and clearly demonstrated that muMRP-14 would not induce human neutrophils to adhere (data not shown). Therefore, assuming a differential species effect, it was decided to locate a murine cell line that could be tested.

4.3.2.1 WEHI 265.1 cell adhesion assays

The first murine cell type to be used in the static adhesion system was WEHI 265.1. As described in section 4.3.1, this cell is a monocyte/macrophage type cell which has been successfully used in chemotaxis investigations of the MRP-14 related protein, MRP-8 (CP-10) (50). Therefore, it was thought suitable as a starting point to investigate the function of MRP-14 in adhesion.

Initial batches of purified MRP-14 were stored in 20mM TRIS-base pH 8 buffer. The buffer was found to stimulate adhesion to varying degrees, with no discernible reason for the variation. However, MRP-14 was found to stimulate adhesion approximately 2.5 times above background levels, induced by the TRIS buffer, used as a control or the Hanks-HEPES buffer, which was used to dilute the protein (data not shown). muMRP-14 did not seem to be as potent an inducer of adhesion as huMRP-14, which generated a >3 times above background increase in adhesion (see Figure 4.2A, on page 139).

This initial data was very hopeful. To avoid TRIS effects in the biological assays, MRP-14 was dialysed into the assay reaction buffer, Hanks-HEPES. Final dialysis of new batches of MRP-14 was henceforth done into Hanks HEPES.

Subsequent results with MRP-14 confirmed the initial pro-adhesive findings (see Figure 4.2B, on page 139). MRP-14 was found to cause a dose-dependent increase in adhesion with a peak response at 3μM, producing 19% adhesion of WEHI cells. This adhesion was not as strong as that induced by 3μM huMRP-14 which produced 27% adhesion. A suitable control, huMRP-6, did not cause any significant adhesion above the background level of 5%. Two features of this assay were the decreased maximal
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adhesion seen with muMRP-14 versus huMRP-14 and also the bell-shaped nature of the muMRP-14 dose-response curve.

A brief investigation was made into the effects that endotoxin-loading had on the proadhesive properties of MRP-14 (Figure 4.3A, on page 140). The protein concentrating effect of endotoxin depletion was controlled for by concentration monitoring and protein dilution. This investigation revealed a potential increase in the proadhesive activity of MRP-14 after endotoxin depletion, although this effect was marginal.

Further comparisons of the potency of muMRP-14 and huMRP14 were performed; a graph from a representative experiment is shown in Figure 4.3B. huMRP-14 was consistently found to be more potent and more efficacious than muMRP-14; that is, huMRP-14 induced more final adhesion and also caused adhesion equivalent to muMRP-14 adhesion at lower doses than those required of muMRP-14. A bell-shaped curve for the pro-adhesive properties of muMRP-14 is again illustrated.

It is not clear what the cause might be for the bell shaped curve of adhesion, induced by MRP-14. Whether it is a detergent inhibition effect at the top concentration used is not clear. To investigate the effect of detergent at high concentrations, MRP-14 was centrifugation-concentrated using Centricons. Use of this system concentrated MRP-14 from approximately 0.5mg/ml up to 5mg/ml; this was associated with aggregation of MRP-14. Laser light scattering investigations (as described in section 3.5.2, on page 124) revealed that the average molecular mass of the MRP-14 protein sample increased from 28±2 kDa to 72±3 kDa (A.Coffer, personal communication). Use of this aggregated protein sample in an adhesion assay gave a maximal adhesion response of 5% with a MRP-14 dose of 3µM (25 times background) versus 90% with non-concentrated huMRP-14 at 4µM (350 times background) (data not shown). This data suggests that the aggregation state of the protein is associated with adhesion function. It also suggests that protein concentration by centrifugation is not the method to investigate the mechanism which may be behind the bell-shaped MRP-14 dose-response curve.

It has been shown that the β2-integrin, Mac-1, is responsible for the adhesion induced by huMRP-14 on human neutrophils (197). Given that both muMRP-14 and huMRP-14 were homologues and generating adhesion with the myeloid cell line WEHI 265.1, it was possible that they would mediate adhesion by the same mechanism. To
investigate this, blocking antibody experiments were performed. These experiments were inconclusive and blocking was not shown with any Mac-1 antibodies (M1/70 and 5C6). Therefore, the identity of the adhesion molecule mediating the pro-adhesive properties of MRP-14 on WEHI 265.1 cells is unknown.

At this stage of the investigation, there was some evidence that MRP-14 was acting in a similar fashion, although at a reduced potency and efficacy, to the human homologue, huMRP-14. Some of the difficulties found with this system included an inability to find a positive control other than huMRP-14 as the cell line did not respond to either fMLP or IL-8. There were also various difficulties in blocking the induced MRP-14 adhesion; this would be a necessary step to definitively prove adhesion and also to dissect out the responding adhesion molecule. Given this data, the decision was taken to investigate the effect of MRP-14 on peripheral resting blood cells, the cell type that had been successfully employed in investigations of huMRP-14.
Figure 4.2:- MRP-14 is proadhesive for the WEHI 265.1 cell line in a static adhesion assay to human fibrinogen. A. MRP-14 (in TRIS buffer) and huMRP-14 but not TRIS buffer cause WEHI 265.1 cells to adhere. ETX- refers to the protein endotoxin depletion that was performed prior to use in the assay. Points are expressed as means of triplicate wells ± SEM in both A and B. B. MRP-14 (in Hanks HEPES buffer) and huMRP-14 but not Hanks HEPES buffer or huMRP-6 cause WEHI 265.1 cells to adhere.
Figure 4.3: A. Endotoxin has a minimal effect on the proadhesive activity of MRP-14. Endotoxin (ETX) removed MRP-14 is a more potent inducer of WEHI 265.1 cell adhesion to human fibrinogen in a static adhesion assay than endotoxin positive MRP-14. Points are expressed as means of triplicate wells ± SEM in both A and B. B. Human MRP-14 is a more potent inducer of WEHI 265.1 cell adhesion to human fibrinogen in a static adhesion assay than murine MRP-14.
4.3.2.2 Purified peripheral cell adhesion assays

Murine neutrophils and PBMCs were purified from murine peripheral blood as described in sections 2.8.1.2 (page 71) and 4.3.1.1 (page 132). PBMCs were relatively free of platelets after 3 centrifugation steps. The results of adhesion assays performed with these cells is shown in Figure 4.4 (on page 142).

Murine neutrophils did not produce a bell-shaped adhesion curve in response to MRP-14 although the pro-adhesive effect was concentration dependent (Figure 4.4A). Maximal adhesion of 30% (2.5 times background) was found at a dose of 5μM MRP-14; this contrasts with the bell shaped adhesion seen with WEHI 265.1 cells in Figure 4.2B (page 139) and Figure 4.3A (page 140). In these cases the high dose showing lack of adhesion was at 6μM MRP-14.

Murine PBMCs also did not produce a bell-shaped dose-response curve to MRP-14 (Figure 4.4B). Maximal adhesion of 44% (<2 times background) was found at a 5μM dose of MRP-14. One of the major problems with the interpretation of this particular assay was the responding cell type, whether this was T-cell, B-cell, monocyte, et cetera.

The decision was taken to move away from resting, peripheral blood cells and into a system that would deliver substantial numbers of peripheral cells; the peritoneal-thioglycollate elicited leukocyte system.
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A.

![Graph A](image1)

Figure 4.4: MRP-14 activates peripheral blood leukocytes to adhere to human fibrinogen in a static adhesion assay. A. MRP-14 but not Hanks HEPES buffer cause purified peripheral murine neutrophils to adhere to human fibrinogen in a static adhesion assay. Points are expressed as means of triplicate wells ± SEM. B. MRP-14 but not Hanks HEPES buffer cause purified peripheral murine PBMCs to adhere to human fibrinogen in a static adhesion assay.

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4.3.2.3 Thioglycollate-elicited cell adhesion assays

Figure 4.5A (page 144) shows the results of a typical adhesion experiment performed with thioglycollate-elicited cells. The maximal adhesion seen in this assay is 4.5% at 1.5μM MRP-14, 3 times background. Although low, this figure is virtually the same for all 3 positive stimuli tested; MRP-14, fMLP and IL-8. All the stimuli generated a titratable dose-adhesion response curve. Interestingly, the bell shaped MRP-14 dose-response curve is again visible with a negative response at 6μM (the same as seen with WEHI 265.1 cells). This experiment suggests that MRP-14 is as potent and efficacious as fMLP and as efficacious as IL-8 (across the concentration range tested).

Monoclonal antibody (mAb) blocking experiments were attempted and a typical example is shown in Figure 4.5B. In this experiment, only 40% of the MRP-14-induced binding could be inhibited with 40μg/ml (4μg dose) or 20μg/ml (2μg dose) of the Mac-1 mAb 5C6, although almost all, 85%, of the binding was inhibitable with the same doses of the Mac-1 mAb M1/70. Similar problems that occurred when trying to block the adhesion of WEHI 265.1 cells also occurred when trying to block the adhesion of thioglycollate-elicited cells in that monoclonal antibodies failed to block in approximately 50% of blocking experiments. Attempts to block a known Mac-1 mediated adhesive event, such as that stimulated by fMLP, with Mac-1, other integrin and control antibodies was also not satisfactory (including attempts with fAb fragments). This was suggestive of either a less-than-100%-Mac-1-mediated adhesion event or incomplete Mac-1 blocking with the antibodies tested. Therefore, the Mac-1 alone data in Figure 4.5B, without important controls such as isotype controls, other adhesion molecule antibodies and other proadhesive stimuli is the best evidence available. Hence, these observations are only preliminary and not conclusive that Mac-1 mediates MRP-14-induced thioglycollate cell adhesion. More investigations must be performed to define the adhesion molecule that mediates the proadhesive effect of MRP-14.
Figure 4.5: A. MRP-14 is proadhesive for thioglycollate elicited leukocytes. MRP-14, fMLP and IL-8 but not Hanks HEPES buffer induce cell adhesion to human fibrinogen in a static adhesion assay. Points are expressed as means of triplicate wells ± SEM in both A and B. B. MRP-14 induced adhesion is partially Mac-1 dependent. The adhesion of thioglycollate-elicited leukocytes, induced by murine MRP-14 can be partially blocked by 40μg/ml of the anti Mac-1 antibody 5C6 and almost abolished by a 40μg/ml of the anti-Mac-1 antibody M1/70 in a static adhesion assay.
4.3.2.4 Further static adhesion assay development

Significant efforts had been directed towards generating good static adhesion assay data. This had been attempted with various cell types and had yielded some interesting results, suggestive of a role for MRP-14 in adhesion. There were 2 major problems with the static adhesion data, the differential of binding (from background to stimulated), which always seemed to be quite small, and the ability to block the adhesion generated by various stimuli with blocking antibodies. With the aim of addressing the major shortfall of binding differential in the assay system, various developmental investigations were performed.

The first set of investigations were concerned with blocking agents. Fish skin gelatin (FSG) is a blocking agent widely used in confocal microscopy. When it was used, in combination with a ligand titration in a WEHI cell adhesion to fibrinogen assay it increased the background (buffer) to stimulated (PdBu) binding index considerably (data not shown) at low ligand concentrations (less than 2µg/ml). This encouraging trend was not repeated with the cell type of choice for the adhesion assay, thioglycollate elicited leukocytes.

Other investigations were concerned with the choice of ligand used on the plate. Human fibrinogen had been used so far as it had been successfully used to mediate human Mac-1 events (5, 197) and did bind murine cells. However, this reaction may not have been optimal. Therefore, other ligands including murine fibrinogen and haemocyanin were used. Haemocyanin is a product from limpets that has been shown to specifically mediate Mac-1 binding (163). However, when using thioglycollate-elicited cells, neither ligand at any concentration tested (from 2mg/ml to 60ng/ml), with or without FSG blocking, produced a stimulated : background binding ratio greater than that seen with the original human fibrinogen at 2mg/ml, without FSG blocking. Therefore, the static adhesion system was not altered.

4.3.3 Chemotaxis assays

The evidence accumulated so far suggested that muMRP-14, like its human homologue, huMRP-14, had a role in the adhesion of neutrophils other leukocytes. Other data accumulated at the same time from in vivo investigations (see section 4.4, page 166) suggested that MRP-14 had a role in myeloid cell migration from the vasculature to the tissues. The next, obvious question to ask was, whether MRP-14 was directly or
indirectly chemotactic? Does MRP-14 signal directly to cells to cause the recruitment seen in vivo, or, alternatively, does MRP-14 signal to an unknown cell which releases an unknown mediator which then causes cell recruitment.

This question was first addressed using a modified version of the human neutrophil chemotaxis assay (198), but here using thioglycollate elicited cells as the migrating cell type (section 4.3.3.1, page 146). Later evidence from in vitro studies (section 4.3.4, page 155) suggested that already migrated cells i.e. thioglycollate cells, may respond differently to resting peripheral blood cells i.e. purified blood neutrophils. This data led to further efforts to develop a chemotaxis assay using peripheral blood cells; these efforts are detailed in section 4.3.3.2 (page 148).

4.3.3.1 Using thioglycollate elicited cells

As has been previously discussed, the main reason for using thioglycollate elicited cells rather than purified peripheral cells was one of animal usage, in that one can obtain 1x10^7 myeloid cells from one animal using thioglycollate-elicited peritonitis (if harvested at 4 hours) versus 4x10^5 neutrophils, if peripheral blood was taken and purified from one animal.

Both the human and murine chemotaxis assays used Transwells to separate the chemotaxin and the responding cells. Transwells are made up of an insert with a polycarbonate filter as the bottom, and also of a base well which is similar to most 24 well tissue culture plates. A schematic diagram of a Transwell is shown in Figure 4.8A (page 154). The next section describes the modifications that were made to the human assay in order to successfully use it with murine cells.

4.3.3.1.1 Initial assay modifications

The human neutrophil chemotaxis assay system uses 5\mu m Transwells and Hanks HEPES buffer supplemented with 0.1% BSA and cations, and measures chemotaxis by FACS scattering and subsequent counting of fully migrated neutrophils (as measured by positioning a gate around neutrophils within the blood leukocyte population), rather than counting cells embedded in the filter. Initial efforts to adapt this system, using 24h thioglycollate elicited cells, which contain more monocytes than the 4-hour sample and up to 3x10^7 lavage cells per animal, were not successful. No chemotaxis to either of the test stimuli, IL-8 or fMLP, was observed (data not shown).
In an effort to generate some form of chemotaxis using myeloid cells, different Transwell pore sizes (8μm, 5μm and 3μm) were tested, different buffer systems including RPMI were tried and also different cell sources, such as 4h thioglycollate elicited cells were used, in the assays. These investigations showed that chemotaxis could be observed using 4h thioglycollate elicited cells in a 3μm Transwell, with either Hanks HEPES or RPMI buffer, fMLP as the stimulus and at an assay timepoint of 1.5 hours (data not shown).

The next step in assay optimisation was choosing a buffer system to use. The same stimuli and cells in either Hanks HEPES or RPMI buffers were measured in the chemotaxis assay. As shown in Figure 4.6A (page 152), RPMI was found to give a maximal response : background (10^-6 M fMLP : buffer) ratio of 7.4 whereas Hanks HEPES produced a ratio of 11. Therefore the assay was subsequently performed in Hanks HEPES.

The final step in assay optimisation was an evaluation of the effect of well coating to migration (Figure 4.6B page 152). The idea was that cells might be sticking to the bare polycarbonate filters, as the assay stood, and therefore the migration seen was may not be an accurate reflection of the 'real' results. Hence, if the wells were coated with a ligand such as fibrinogen, this might aid migration. This was borne out by the results, although the degree of improvement was not large. Maximal response : background ratio was 8 for the coated wells and 6.3 for the uncoated wells. However, the IL-8 response was better in the uncoated wells. Taken together, the assay seemed to be optimal when uncoated wells were used. To check assay functionality further, equal fMLP concentrations were added to the top and the bottom wells. As expected, this blocked cell migration, suggesting that the assay was specific and was measuring chemotaxis.

4.3.3.1.2 Is MRP-14 chemotactic?

Many assays with different recombinant protein batches were performed, to assess whether or not MRP-14 was chemotactic. The concentration range chosen to assay this question was designed to take into account the optimal adhesion concentration of MRP-14 (1.5μM, see Figure 4.5, page 144) and also the reported maximally chemotactic concentration of MRP-8 (10^-13 M) (137).
An observation from the in vivo arm of the MRP-14 study suggested that non-endotoxin depleted protein would recruit more cells than endotoxin-depleted protein (data not shown). To investigate whether this effect was direct or indirect, endotoxin-rich and -depleted MRP-14 were tested in the chemotaxis assay (Figure 4.7A, page 153). This investigation suggested that neither the presence of endotoxin nor detergent (present to a degree in the endotoxin depleted samples) in MRP-14, nor MRP-14 itself, caused direct chemotaxis of thioglycollate-elicited cells over a concentration range from 2μM to 2 x 10⁻¹²M. In contrast, fMLP at 1μM at 0.1μM and IL-8 at 30nM induced chemotaxis at approximately 4 and 6 times buffer induced migration, respectively.

Further investigations, using a very wide concentration range from 3x10⁻⁶M to 3x10⁻²⁰M, were performed (data not shown). This set of experiments confirmed that MRP-14, was not directly chemotactic for thioglycollate-elicited leukocytes.

4.3.3.2 Using peripheral cells

Later data obtained from calcium flux investigations (section 4.3.4, page 155) suggested that there was a functional difference between thioglycollate-elicited cells and cells isolated from peripheral blood. This data led to the question of whether the conclusion that MRP-14 was not directly chemotactic, drawn from experiments which made use of pre-activated, pre-migrated myeloid cells, was true in the in vivo situation where the responding neutrophils are resting and not pre-activated.

Unfortunately, this question was very difficult to answer. Indeed, the reason that in vitro chemotaxis and adhesion were investigated using thioglycollate-elicited cells was the small number of peripheral neutrophils that could be obtained from a single mouse.

Two systems to test the hypothesis that MRP-14 might be chemotactic for peripheral neutrophils were investigated. These were the modified Dunn chamber (a slide based method) and also a new Boyden-like, 96 well chamber (Neuroprobe). The results of these investigations are detailed below.

4.3.3.2.1 Modified Dunn Chamber

The modified Dunn chamber chemotaxis assay is a relatively new chemotaxis assay (290) (method described in detail in (289)). It is a slide based assay system (represented in Figure 4.8B, page 154) consisting of an inner and outer well, separated by an optically
polished area, which allows observation of cell movement and also creates an area in which the chemotactic gradient can form. The gradient is formed across this polished area by placing the chemotactic agent in the outside well and buffer in the inside well. Cells are either grown on, or allowed to adhere to, the coverslip and this is placed on the chamber and sealed with wax. During assay set up, the inner and outer wells are filled with buffer and the coverslip added so that there is a gap above a portion of the outer well. This allows buffer removal and the addition of the chemotaxin, which floods the outside well and allows contact with the buffer containing polished region. Cells can then be observed moving across the optically polished area using time lapse video microscopy and computer analysed, if necessary. This system has been successfully used to observe the movement of human neutrophils (291).

Many attempts were made to optimise and generate results with this system. The first major stumbling block was a purely technical one, that of ensuring the unit was adequately sealed with wax, in order to prevent evaporation of solutions during the experiment. Once this was overcome, the second major problem was finding cells in the optically polished area to observe. Obviously, only a portion of the polished area could be observed during the experiment, so an area that contained cells was chosen. The assay is designed to run as a slide might usually be observed under a microscope, with the coverslip uppermost. As non-activated peripheral neutrophils do not adhere to the coverslip, even when it is coated with FCS, the majority fall to the bottom of the inner and outer wells upon assay set-up. They are hence no longer within the focal range of the microscope. In an attempt to overcome this problem, the assay was inverted so that neutrophils would fall onto the coverslip which would allow their observation.

To further maximise the number of cells that could be observed, cells were added with buffer to the inside well whilst buffer alone was added to the outside well. When the coverslip was added and sealed, the outside well fluid was removed by capillary action onto a tissue and cells were drawn from the middle of the chamber, into the polished area. The outer well medium was then replaced by chemotaxin as well as cells, to equalise cell density throughout the chamber, and obviate any chance migration that may be due to a so called ‘entropy’ effect (where cells might move from the centre because they wanted to be in a less densely populated area (D.Zicha, personal communication)). A total of $2 \times 10^5$ cells were added to each chamber.
These modifications, including assay inversion, allowed the observation of a number of cells. Indeed, up to 40 cells could be observed in one field at the start of an experiment. Initially, the system seemed to generate some good data after a 3 hour incubation at 37°C. Chemotaxis, the actual movement of cells from the buffer region to the chemoattractant could be observed with fMLP and tantalisingly, with $10^8$M MRP-14, although not with the buffer control (video data not shown). Unfortunately, when attempts were made to block this migration by placing chemoattractant in both wells, the system started to show certain artefacts, e.g. cells moving under blocking conditions, but towards the inner well and also movement in response to buffer control (video data not shown).

Due to such apparently basic problems with the modifications that had been made to the system and an inability to use the proven original system, further investigation was suspended. Other disadvantages with the modified and original systems included the inability to perform assays with more than 6 conditions at once (limited to the number of time-lapse video microscopes available, which made titrations and duplicates very difficult) and the limitation of observing only a small percentage of the cells rather than the complete population (as is not the case in other assay types, such as the Transwell system).

4.3.3.2.2 Neuroprobe Chamber
The second system in which \textit{in vitro} chemotaxis of peripheral neutrophils was evaluated was the Neuroprobe non-disposable chamber. This chamber was analogous in design to the Transwell but technically more challenging to use as the basic assay set up consisted of a 96 well plate. To each well was added sufficient sample volume so that a positive meniscus was formed, above the surface of the well. To this was added a 96 well platesized 3μm polycarbonate filter, which was covered by a 96 well rubber gasket and then everything was held down by pegs, in a chamber which enclosed all the components. The top of the chamber contained wells which contacted with the polycarbonate membrane and hence the chemotactic stimuli in the wells below. Cells could then be added to the top wells. As the positive meniscus had already 'wet' the filter, buffer exchange and cell movement could take place.

There was no direct way to measure cell movement, so cells were labelled with BCECF-AM or green cell tracker (GCT) dye. Cell counting by FACS scattering was
not considered a viable option as there were simply not going to be enough migrated cells to count (approximately 16,000 cells were added per well). Unfortunately, not one experiment in the whole series was successful. In the majority of experiments, one or all of the controls failed and triplicate wells confirmed the noise inherent in the system. Later experiments suggested that BCECF-AM might be inhibitory to cell migration as there was increased migration with GCT labelled cells versus BCECF labelled cells (data not shown) but even this migration was not optimal.

In summary, the signal:noise ratio of the Neuroprobe system was too low and there were no repeatable phenomena. This suggested that no pattern or phenomena existed. Given the problems found with both the Modified Dunn and the Neuroprobe chambers to evaluate peripheral chemotaxis, it remains to be seen whether MRP-14 is directly chemotactic for peripheral neutrophils.
Figure 4.6: A. Comparing the effects of Hanks HEPES and RPMI buffers in a 3μm Transwell model of chemotaxis using 4h thioglycollate-elicited leukocytes. For both A. and B. points are expressed as means of duplicate wells ± SD. B. Comparing the effects of coating Transwells with fibrinogen in a Hanks HEPES buffered, 3μm Transwell model of chemotaxis using 4h thioglycollate-elicited leukocytes.
Figure 4.7: Endotoxin does not induce chemotaxis. MRP-14 ETX+, Hanks HEPES ETX+, or endotoxin removed counterparts do not induce chemotaxis of 4h thioglycollate elicited leukocytes although IL-8 and fMLP do induce chemotaxis in a 3μm Transwell model of chemotaxis. Points are expressed as means of duplicate wells ± SD.
Figure 4.8 - Various apparati used to evaluate chemotaxis. A. Schematic diagram illustrating the CoStar Transwell used to evaluate chemotaxis. B. Schematic diagram illustrating the modified Dunn chamber used to evaluate peripheral cell chemotaxis.
4.3.4 Calcium flux assays

The \textit{in vitro} data collected so far suggested that MRP-14 was proadhesive for various cell types, including neutrophils but was not directly chemotactic for the same cells (with the caveat, it is assumed that recruited cells have the same chemotaxin sensitivity as peripheral, resting cells). More evidence was sought to indicate whether or not MRP-14 was able to stimulate leukocytes. One general marker of cell activation is calcium flux. Indeed, calcium flux has been shown to occur in neutrophils, after the engagement of the $\beta_2$-integrin, Mac-1; this fluxing was localised to the sites of local Mac-1 ligation (204). However, calcium flux is not necessary for neutrophil effector functions such as degranulation or oxidative burst (235). The results from two methods of evaluating the effect of MRP-14 on calcium flux will be presented in this section, the cuvette method (used to analyse cell populations) and the confocal microscopy method (used to analyse small populations or individual cells).

4.3.4.1 Analysing whole populations

In this method, the response of a whole population of cells, rather than a sub-population, or even single cells is examined. A cell population were labelled with the fluorescent calcium dye, Fluo-3-AM. This dye absorbs light at 485nm and only emits light at 535nm when it is bound to calcium. As the dye has been taken up by the cells and is intracellular, a positive signal corresponds to either an influx of calcium from ion channels or release from intracellular stores. As this method measures the fluorescence of the whole population, the results must be interpreted carefully. For example, a strong response may indicate a large proportion of the cells responding weakly and a weak response as a smaller proportion responding strongly, rather than all the cells responding in an equivalent fashion.

As has been common in the \textit{in vitro} investigations of MRP-14 function, the first cell type to be evaluated in this system were the 4h thioglycollate-elicited leukocytes. The results of this investigation are shown in Figure 4.9 (page 159). Figure 4.9A shows that 0.1\textmu M fMLP but not 0.1\textmu M MRP-14 generated a calcium flux. To demonstrate that a potential MRP-14 response was not being inhibited by an initial fMLP mediated effect, 1\textmu M MRP-14 was applied before 0.1\textmu M fMLP (Figure 4.9C). This demonstrated that MRP-14 did not generate a calcium flux, and that this lack of action was not affected by pre-stimulation of the cells with fMLP. Furthermore, the data was
suggestive that MRP-14 was not contaminated with fMLP or other bacterial peptides as the fMLP response in this situation was comparable with that when cells are stimulated alone with fMLP (Figure 4.9A).

It was observed in Figure 4.9C that the application of 1μM MRP-14 caused a drop in the baseline fluorescence of MRP-14 which recovered slowly, over the course of almost 12 minutes. To test whether this was a buffer effect (given that 1μM MRP-14 was 10% total volume of the cuvette) rather than a specific internal, calcium-sequestration effect, an equivalent amount of buffer was applied (Figure 4.9B). This caused a virtually identical effect, suggesting that the effect was indeed buffer mediated. As neither 1μM Hanks HEPES (Figure 4.9B) nor 1μM MRP-14 (Figure 4.9C) prevented a subsequent 0.1μM fMLP response, it may be concluded that neither the buffer, nor MRP-14 were toxic to the cells, over the course of the experiment.

It was of interest to know whether the amount of endotoxin that was present on undepleted MRP-14 was capable of activating cells directly. This was tested by the application of 1μM MRP-14 ETX+ (Figure 4.9D) followed by 0.1μM fMLP. As was found in the other experiments, MRP-14 did not generate a calcium flux, nor did it prevent the action of a further fMLP stimulus. This data suggests that the amount of endotoxin present on undepleted MRP-14 did not activate cells over the course of the experiment (20 minutes).

The second set of experiments that were performed were with peripheral leukocytes, generated by dextran mediated erythrocyte sedimentation of whole blood. The remaining cell population was then labelled with Fluo-3 and used in the whole population (cuvette) calcium flux assay.

The findings of this set of experiments were a considerable surprise. Figure 4.10A (page 160) shows that the application of 0.1μM fMLP generated the expected calcium flux. However, the application of 0.1μM MRP-14 caused a flux reaction of equal magnitude to the fMLP response, but of an extended duration, with a very slow decay, to the 20 minute termination. This was in stark contrast to the response of thioglycollate-elicited cells (Figure 4.9A). That this MRP-14 induced reaction was not dependent on fMLP pre-stimulation was subsequently demonstrated in Figure 4.10B. This data also suggested that if the cells that were responding to fMLP were the same cells as had responded to MRP-14, then they had experienced an increased intracellular calcium concentration from MRP-14 and were still responsive to fMLP.
That the MRP-14 response was not mediated by a buffer component was demonstrated in Figure 4.10C; 0.1μM Hanks HEPES did not generate a calcium flux (0.1μM buffer is the amount of buffer that 0.1μM MRP-14 was contained in). The threshold of the MRP-14 response was then sought. Figure 4.10C also shows that 10nm MRP-14 was insufficient to generate a calcium flux and that a supra-threshold dose of 1μM did not generate a larger response than that seen with 0.1μM MRP-14. This suggested that the threshold concentration to produce a MRP-14 induced calcium flux was between 10nm and 100nm and also that 0.1μM MRP-14 calcium flux was maximal.

This section has demonstrated a dichotomy in the ability of MRP-14 to generate a calcium flux response. MRP-14 caused peripheral cells but not pre-recruited neutrophils and monocytes to undergo calcium flux. It may be that the responding peripheral cell type was neither a neutrophil or monocyte, which would be one explanation for the non-response of the thioglycollate elicited cell. However, this difference may also have represented a difference in the ability of MRP-14 to stimulate myeloid cells, based on their activation state prior to MRP-14 stimulation.

To resolve this question, further experiments were performed with isolated peripheral cell types. For reasons already elucidated, these experiments were not repeated on the relatively large scale of the cuvette model, but rather utilising a novel method for observing the calcium flux in a few hundred cells, developed at the ICRF.

4.3.5 Identification of responsive cell types using the confocal microscope

The method employed to dissect out the responding cell type from a total population of peripheral leukocytes was the observation of calcium flux using the confocal microscope. Peripheral blood was depleted of erythrocytes and separated into neutrophil rich and mononuclear cell fractions using the Percoll gradient method. These fractions were labelled with Fluo-3 and stuck down to glass bottomed plastic dishes that had been coated with poly-L lysine. These cells were then observed under the microscope before and after stimulation and the responses recorded as a set of graphic frames which were later assembled into films. Frames from the start and end of the films are shown in the figures below.

In all the figures the confocal images are of low resolution. This was deliberately done to maximise the number of cells observed, particularly important in the multi-
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member mononuclear cell population. Figure 4.11 (page 161) shows a set of confocal images recorded from neutrophils. Before all treatments there was a period of recording to assess the baseline fluxing. Figure 4.11E shows that there was no overall change in cellular fluorescence when the cells were not stimulated. Figure 4.11A shows that the addition of 0.1μM buffer did not affect the cells. In Figure 4.11B, 0.1μM fMLP can be seen to have caused an increase in the frequency of fluorescence of the cells (seen as an increased number of fluorescent cells) and a small generalised increase in cellular fluorescence. Figure 4.11D shows that the addition of 0.1μM MRP-14 caused an increase in the frequency of fluxing as well as a generalised increase in cellular fluorescence. A similar pattern existed in Figure 4.11C, with the application of 1μM MRP-14.

The images obtained from the mononuclear cell preparations were significantly different in appearance to the neutrophil images. The major difference was a multitude of platelets that did not seem to be fixed to the dish, as the other cells were. Observation of cells without treatment showed that the baseline was stable (data not shown). Figure 4.12A (page 162) shows that although there was a high background fluxing, the addition of 0.1μM buffer had no effect. 0.1μM fMLP (Figure 4.11B) caused a general increase in the frequency of fluxing but no effect on general cellular fluorescence. In contrast, treatment with 1μM ionomycin resulted in an increase in general fluorescence (Figure 4.11E). The most interesting effects were observed with 0.1μM (Figure 4.11C) and 1μM (Figure 4.11D) MRP-14. Addition of either concentration of MRP-14 caused a rapid increase in general cellular fluorescence, accompanied by increase in flux frequency and an apparently strong platelet effect. Closer observation of this platelet phenomena suggested that the platelets were aggregating. Further identification (whether T-cell, B-cell, monocyte et cetera) of the cells fluorescing, excepting the platelets, was not possible.

This investigation was initiated to dissect the responding cell type(s) that respond to MRP-14. The data suggests that neutrophils, platelets and one or more of monocytes, lymphocytes, eosinophils and basophils are also responding. This suggests that MRP-14 is a generalised peripheral leukocyte activator.
Figure 4.9: MRP-14 does not induce calcium flux in thioglycollate elicited leukocytes, using a cuvette calcium flux model. A. 0.1μM fMLP but not 0.1μM MRP-14 cause calcium flux. B. 1μM Hanks HEPES caused a baseline drop whilst 0.1μM fMLP causes a calcium flux. C. 1μM MRP-14 causes a baseline drop whilst 0.1μM fMLP causes a calcium flux. D. 1μM endotoxin-positive-MRP-14 causes a baseline drop whilst 0.1μM fMLP causes a calcium flux.
Figure 4.10: MRP-14 does induce calcium flux in peripheral blood leukocytes, using a cuvette calcium flux model.  

A. Both 0.1uM fMLP and 0.1uM MRP-14 cause calcium flux.  
B. Both 0.1uM MRP-14 and 0.1uM fMLP cause a calcium flux.  
C. 0.1uM Hanks HEPES and 10nM MRP-14 did not cause but 1uM MRP-14 did cause a calcium flux.
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<td>0.1uM MRP-14</td>
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Figure 4.11: MRP-14 causes calcium flux in peripheral neutrophils. Figures are numbered 1, for a pre-treatment exposure and 2, for a post treatment exposure. A. 0.1uM Hanks HEPES does not cause calcium flux. B. 0.1uM fMLP does cause calcium flux. C. 1uM MRP-14 does cause calcium flux. D. 0.1uM MRP-14 causes calcium flux. E. When no treatment is applied, no calcium flux is observed.
Figure 4.12: MRP-14 causes calcium flux in peripheral mononuclear leukocytes. Figures are numbered 1, for pre-treatment exposure and 2, for post treatment exposure. A. 0.1uM Hanks HEPES does not cause calcium flux. B. 0.1uM fMLP does cause calcium flux. C. 1uM MRP-14 does cause calcium flux and apparent platelet aggregation. D. 0.1uM MRP-14 causes calcium flux and apparent platelet aggregation. E. 1uM ionomycin causes calcium flux.
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4.3.6 Superoxide assays

The neutrophil oxidative burst is a hallmark of the fully activated cell and results in the destruction of phagocytosed bacteria. The oxidative burst is mediated by the gp$_{phox}$ system, driven by NADPH reduction to NADP$^+/H^+$ which converts 2 oxygen molecules to superoxide (O$_2^-$) molecules. These superoxide molecules can then interact with hydrogen peroxide and the enzyme myeloperoxidase to generate the highly toxic HOCl$^-$ and ClO$^-$ molecules. In combination with other granule contents, released into the phagosome, the oxygen radical species can attack the bacteria, puncturing cell walls and thereby causing death (reviewed in (44)).

The in vitro data accumulated so far suggested that MRP-14 played a role in adhesion, but not chemotaxis and that it might stimulate naïve rather than pre-activated cells. Another important question was whether MRP-14 causes oxidative burst in the neutrophil. The chemotactic MRP-14 analogue, MRP-8 does not cause superoxide burst (56) although other chemoattractants such as fMLP, do.

As with previous investigations, naïve and activated leukocytes were assayed for this activity to assess whether there was a functional dichotomy in the cell types. Peripheral leukocytes were prepared by simple erythrocyte sedimentation and the results analysed by gating on the neutrophil population. Figure 4.13A (page 165) shows that MRP-14, from 2µM to 0.1µM, does not induce superoxide generation in peripheral leukocytes. Non-endotoxin depleted MRP-14 did not cause superoxide generation either (data not shown). However, 1µM fMLP and 10pM MIP-2α, the murine IL-8 homologue (CXC chemokine), did generate superoxide burst, of 160% and 125% buffer control, respectively.

The situation was broadly similar with the thioglycollate cell preparation (Figure 4.13B). In this situation, both MIP-2α and MRP-14 appeared to generate a control fluorescence 10% higher than control values although this is probably not significant, especially in the light of the increased response differential between negative and positive control, versus the peripheral cells. These stimuli were hence considered not to induce superoxide burst. 1µM fMLP was still able to generate superoxide burst, of 210% control levels.

This data suggests that MRP-14 does not induce superoxide burst in either peripheral or pre-recruited cells. In contrast to the in vitro data from the calcium flux experiments, MRP-14 did not affect peripheral and pre-recruited cells differently,
suggesting that if it shown to be directly chemotactic, it would not belong to either the
classical or pure classes of chemoattractants. Classical chemoattractants cause
chemotaxis, calcium flux and superoxide burst whereas non-classical chemoattractants
cause chemotaxis without calcium flux, or superoxide burst (90). This work was carried
out by Philippe Tessier as part of the collaborative effort to characterise the function of
MRP-14.
Figure 4.13:- A. MRP-14 does not, but fMLP and MIP-2 alpha do, induce superoxide generation in peripheral leukocytes. For both A. and B. points are expressed as average percentages of control fluorescence ± SD. Number of experiments for each series is shown in the legend. B. MRP-14 and MIP-2 do not, but fMLP does, induce superoxide generation in thioglycollate-elicited leukocytes.
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4.4 *In vivo* investigations

The investigation into the function of MRP-14 was performed in 2 main areas, *in vitro* and *in vivo*. This section characterises the *in vivo* aspect of the investigation. As has been mentioned previously, the *in vivo* investigations into the function of MRP-14 were carried out to elucidate the *in vivo* role of MRP-14, and hopefully to shed some light, by inference, onto the *in vivo* function of huMRP-14 (something which could not be studied directly). It is worth mentioning that, although the *in vitro* and *in vivo* investigations are described separately in this chapter, they were carried out almost simultaneously in the laboratory and obviously the results from one section impacted on the second, and *vice versa*.

The model chosen to examine the *in vivo* function of MRP-14 was a well established method that has been generally termed ‘an *in vivo* model of chemotaxis’ (53). The air pouch model (see Figure 4.14A, on page 171) is basically one in which a vacuole is induced beneath the skin on the back of a mouse by the injection of sterile air. Into this vacuole is then injected a stimulus; the response from the stimulus is measured by sacrificing the mouse and washing out the pouch at a set time point. Cells that may have emigrated towards the stimulus, into the pouch can then be counted and analysed. This method is widely used and the particular version used in the laboratory was described in 1997 (256) and based on earlier work (69).

All *in vivo* work was carried out as a collaboration with Matthew Robinson and Philippe Tessier.

4.4.1 Air pouch assays

The investigations into the *in vivo* function of MRP-14 using the air pouch model, were split into 3 main areas: establishment of a response to the protein and dose-response titration, kinetic response, and specificity investigations. These investigations were not carried out sequentially but rather some investigations in each direction were pursued before definitive investigations in each of the areas were performed.

4.4.1.1 Dose response

Preliminary investigations into the *in vivo* response induced by MRP-14, using only a few doses of protein suggested that MRP-14 was highly active in the air pouch model at
6 hours (data not shown). Later investigations continued using the 6 hour endpoint as this had already generated a strong response in the mice.

MRP-14 was titrated in the air pouch model, from 100μg, down to 0.1μg. Within any given experiment, the specificity of response was only monitored by the use of buffer as negative control, in addition to TNF-α as a positive control. The limited number of controls used was due to the limiting number of animals available for each experiment allied to the amount of work involved. Further specificity investigations were carried out at a later time.

Stimuli were administered to the air pouches as a 1ml dose of PBS, containing the stimulus and cations (1mM Ca++, 1mM Mg++, and 10μM Zn++). Buffer control stimuli were generated according to the peak dose of protein used. For example, if the peak protein dose was 100μg, and this was obtained from adding 100μl endotoxin-depleted protein (kept in Hanks HEPES) to 900μl PBS plus cations then the buffer control would be administered as 100μl endotoxin depleted Hanks HEPES plus 900μl PBS plus cations.

A summary of the results obtained from the dose-response investigation of MRP-14 is shown in Figure 4.14B (page 171). The peak response in the air pouch model was found to be 50μg. The general pattern of response was bell shaped, from which the response tailed off at <1μg. The tail off at the higher doses occurred at 100μg.

At the peak response dose of 50μg MRP-14, the infiltrating cell population was made up of 70% neutrophils (as marked by monoclonal antibody 7/4) and 30% macrophages / monocytes (as marked by monoclonal antibody F4/80) (see Figure 4.17A, page 174). The demographics of this population did not change with the titration of MRP-14 and hence the size of the induced influx (data not shown). TNF-α was also found to induce an infiltrate of 30% monocytes / macrophages and 70% neutrophils although the buffer control contained 70% monocytes/macrophages and 30% neutrophils (see Figure 4.17A).

Higher doses than 100μg were not given as there appeared to be a bell-shaped response, suggesting that the experiment would not generate a response. Furthermore, protein concentration had become an limiting factor in this assay as well as various other assays, such as adhesion assays and therefore protein concentration steps would be required to achieve doses greater than 100μg. This has been found to be associated with
protein aggregation and a loss of activity (section 4.3.2.1, page 136). Finally, it was noted that when doses greater than 100μg were added to cation-laden PBS, a degree of precipitation occurred, although whether this was protein or cation complexes, was not elucidated.

4.4.1.2 Kinetics
Once it was established that the dose giving a peak response at 6 hours was 50μg, the next investigation was to investigate the kinetics of the induced inflammatory response. This was done both to check that the peak response to 50μg MRP-14 was at 6 hours and also to profile the infiltrating cell population in terms of lineage. In each of the kinetic experiments performed, between 4 and 5 mice were used for each data point.

Figure 4.15A and B (page 172) summarise the kinetic data obtained from the use of 50μg MRP-14, 1μg LPS, 1μg murine MIP-2 and Hanks HEPES in the air pouch. In both types of kinetic experiments, MRP-14 was found to generate a peak response at 6 hours which had returned to almost background levels by 12 hours. Data shown in Figure 4.15B suggests that the MRP-14 response is sustained at 9h and resolves in the period between 9 and 12 hours. In contrast to the response elicited by MRP-14, LPS (Figure 4.15A) was found to induce a slower acting response, which peaked at 12 hours and left a background inflammation at 24h and 48h. This suggested that MRP-14 was not acting as a pro-inflammatory agent as result of LPS contamination. MIP-2, the direct acting chemokine, was found to cause maximal inflammation at 2 hours, which was resolved by 6 hours (Figure 4.15B). As MRP-14 peaked at 6 hours, 4 hours later than the peak of MIP-2, the direct acting chemokine, this suggests that MRP-14 may not be acting directly to recruit cells, a hypothesis supported by the negative in vitro chemotaxis data.

When the peak cellular infiltrate was analysed, MRP-14 (6h) and MIP-2 (2h) were found to recruit 70% neutrophils and 30% monocytes / macrophages (Figure 4.17A, page 174). In contrast, LPS (12h) was found to elicit 95% neutrophils and 5% monocytes / macrophages (Figure 4.17B) although LPS (6h) generated 50% monocytes / macrophages and 50% neutrophils (data not shown).

In summary, kinetic investigations of the pro-inflammatory action of MRP-14 suggest that MRP-14 acts more slowly than the direct-acting chemokine, MIP-2. The data also suggest that MRP-14 acts more quickly than the indirect stimulant, LPS.
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Taken together, this suggests that MRP-14 is acting as a chemoattractant, in an indirect fashion, but not via the same mechanism as LPS.

4.4.1.3 Thrombin contamination

As has been mentioned previously, thrombin contamination was found in batches of MRP-14, before treatment with anti-thrombin III agarose beads was introduced into the protocol (section 3.5.3, page 125). The majority of the MRP-14 air pouch data was obtained using protein prepared using the original method that did not remove carried-through thrombin. Therefore, it was imperative to discover whether MRP-14 or thrombin, or a combination of the two was generating a response in the air pouch model.

In an attempt to answer this question, newly synthesised, thrombin-free MRP-14, along with 10 week old, thrombin-laden MRP-14 were evaluated in the air pouch system. As can be seen in Figure 4.16B (page 173), 50µg of thrombin-free MRP-14 was able to induce a inflammatory response in the air pouch, to levels which are similar to the study average for 50µg MRP-14 (Figure 4.16A). 10 week-old thrombin laden MRP-14 generated a small inflammatory response, approximately 50% of that induced by thrombin-free new-MRP-14 and 200% of buffer induced inflammation. Thrombin activity within the contaminated batch of MRP-14 was confirmed with a chromogenic assay (as described in section 2.6.4.2.2, page 69) prior to assay initiation. A sample of newly generated MRP-14 which was thrombin contaminated was not tested.

As pro-inflammatory activity was seen in both the thrombin-free and thrombin laden protein samples, and the thrombin in the second MRP-14 sample was active, this suggests that the pro-inflammatory activity seen in the air pouch is due to MRP-14.

In summary, this preliminary data suggests that MRP-14 and not thrombin contamination is responsible for the pro-inflammatory activity of MRP-14, seen in the air pouch model of inflammation. This data requires further confirmation as the experiment was performed only once.

4.4.1.4 MRP-14 specificity

The data presented so far have suggested that MRP-14 is an agent that induces a inflammatory response in the mouse air pouch model. The maximal MRP-14 induced inflammation occurred at 6 hours after a 50µg dose. This was dissimilar to the direct acting chemokine MIP-2, which acts maximally at 2 hours and also dissimilar to LPS,
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which acts maximally at 12 hours. The final question concerned the specificity of the MRP-14 induced inflammation.

Several control stimuli were used in the air pouch, in an effort to demonstrate the specificity, or otherwise, of MRP-14. Figure 4.14B (page 171) shows that a human S100 family member, huMRP-6, which was produced in a similar fashion from *E. coli* (M. Robinson, unpublished data), and is kept in the same buffer as MRP-14, does not induce an inflammation at any of the doses tested. Given the relative homology between the S100 family members, the fact that MRP-6 did not induce inflammation in the air pouch strongly suggests that the MRP-14 response was specific.

Another control recombinant protein, His-p47 was evaluated in the air pouch model, along with its buffer as a control. p47 is a component of the p97 membrane-fusion pathway (132) and was chosen because of its availability at the ICRF (produced by A. Coffer, ICRF). 50μg p47 did not produce an inflammation above the standard buffer control, or its own buffer control (data not shown).

When another S100 protein was tested in the air pouch model, bovine S100A, at a dose of 50μg, an appreciable inflammation was induced of twice background levels. However, this inflammation was 50% of that induced by MRP-14, although it was 300% of buffer control (data not shown).

When all the 6 hour data generated from the air pouch study was pooled and analysed (Figure 4.16A, page 173), MRP-14 was seen as a potent, specific, inflammatory agent. 50μg MRP-14 was as potent as 1μg LPS and approximately twice as potent as 50ng TNF. The inflammation induced by MRP-14 in the air pouch model was deemed specific as control proteins including the closely related family member, huMRP-6, and buffer controls did not induce a similar inflammatory reaction.
Figure 4.14: MRP-14 induces a profound cellular infiltrate into the air pouch with a maximal response at a dose of 50μg. **A.** Schematic representation of the air pouch method. **B.** MRP-14 induces a peak inflammatory response at a dose of 50μg. Summary of dose-response titrations of MRP-14 and huMRP-6 in the air pouch model. Cells were harvested 6 hours after introduction of stimulus into the pouch. The relative composition of Hanks HEPES varied depending on the peak concentration of protein used and is shown as 150μg rather than the true 100μg for clarity. Data points are represented as means ± SEM (total n=3-34 from 1 to 10 experiments).
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A.

![Graph](image)

**Figure 4.15:** The pro-inflammatory effect of MRP-14 peaks at 6 hours versus 12 hours with LPS and 2 hours with MIP-2. **A.** MRP-14 acts more quickly than LPS, causing a peak recruitment at 6 hours versus 12 hours, respectively. Investigation of recruitment kinetics in the air pouch. Cells were harvested at varying times after introduction of stimulus into the pouch. Data points are represented as means ± SEM (n=3-5 from 1 experiment). **B.** MRP-14 acts more slowly than MIP-2, causing a peak recruitment at 6 hours versus 2 hours, respectively. Summary of recruitment kinetics in the air pouch. Cells were harvested at varying times after introduction of stimulus into the pouch. Data points are represented as means ± SEM (n=8 from 2 experiments).
Figure 4.16:- The pro-inflammatory action induced by MRP-14 is specific and is not mediated by thrombin contamination. A. MRP-14, LPS and TNF, but not huMRP-6 or Hanks HEPES buffer control induce a strong inflammatory reaction in the air pouch. The relative composition of Hanks HEPES varied depending on the peak concentration of protein used. Data points are represented as means ± SEM (total n=3-34 from 1 to 10 experiments). B. Thrombin contamination is not responsible for the pro-inflammatory effect of MRP-14, seen in vivo. Newly synthesised, thrombin-free and 10 weeks old thrombin-laden MRP-14 are both pro-inflammatory in the air pouch model. Data points are represented as means ± SEM (n=3, from 1 experiment).
Figure 4.17: FACS profiling of infiltrating cells in the air pouch model.

A. Neutrophil (7/4) and monocyte / macrophage (F4/80) FACS profiles of cells infiltrating the air pouch after 6 hours, induced by buffer control, TNF and MRP-14. B. Neutrophil (7/4) and macrophage / monocyte (F4/80) FACS profiles of cells infiltrating the air pouch after various times. MIP-2 profiles were obtained after 2 hours and LPS profiles were obtained after 12h.
4.5 Discussion

4.5.1 Peripheral cell purification

A method to purify murine neutrophils from peripheral blood has been developed from a pre-existing human-neutrophil purification method. It was found that separation of dextran sedimented blood, (made up of 20% T-cells and 44% neutrophils) over a 64% : 74% Percoll step gradient would produce a top layer rich in mononuclear cells at the surface of the Percoll gradient and a Percoll included layer rich in neutrophils, found at the 64% : 74% interface (5% T-cells, 74% neutrophils). The relative purity of the neutrophil-rich fraction appears to be low, especially when it is considered that 84% of all the cells in the sample are in the neutrophil region of the scatter plot and 10% of all cells in the sample are present in the lymphocyte area of the scatter plot (see Figure 4.1A, page 135). However, these statistics could not be improved by manipulation of either the sedimentation or the Percoll gradient characteristics. A possible reason why the percentage of neutrophils, 74%, identified by the 7/4 antibody, does not match the 84% of total cell counts found within the neutrophil area, may be that there are contaminating eosinophils. Unfortunately, this could not be tested by FACS staining as an murine eosinophil specific antibody could not be sourced.

One source of mononuclear cell contamination, in the neutrophil-rich fraction may have originated from the layer harvesting. Inevitably, not all the mononuclear cells are harvested from the top layer before harvesting from the neutrophil rich layer begins. Pipette contamination via layer disturbance may have contributed to this.

Despite the problems associated with this method, 74% neutrophil purity was enough to work with in adhesion and calcium flux assays. However, any conclusions about a small percentage of responding cells, derived from the use of this method must be tempered by the possibility that a cell type other than the neutrophil may be mediating the reaction.

Apart from resting peripheral blood cells, obtained from peripheral blood, the other major cell population used in these investigations was derived from thioglycollate-induced peritonitis, harvested at 4 hours, and comprised of 12% macrophages / monocytes, 62% neutrophils, 10% B cells and 7% T cells. It is thought that the thioglycollate-derived cells were not contaminated with blood, as adjudged by the absence of erythrocytes in the peritoneal lavages. Therefore, the lymphocytes must be derived from pre-resident (such as CD5 B-cells) or truly migrated cells. Abortive
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Attempts were made to further purify this population by separation over the 64% : 74% Percoll gradient. It remains a tantalising prospect to try and further purify this population to neutrophil homogeneity.

4.5.2 Adhesion

This chapter has presented evidence for a role of MRP-14 in the adhesion of several cell types, including naïve and activated neutrophils, naïve mononuclear cells and the WEHI 265.1 monocytic cell line, to fibrinogen.

The adhesion induced by MRP-14 was specific. In the WEHI 265.1 cell system, MRP-14 and huMRP-14 could induce adhesion but the closely related S100A family member, huMRP-6 was unable to induce any adhesion (Figure 4.2B, page 139). The same findings were repeated in the thioglycollate system with the additional positive controls of fMLP and IL-8 (Figure 4.5A, page 144) although huMRP-6 was never tested (full attention was switched to blocking). In the least characterised cell type, peripheral neutrophils and mononuclear cells, only muMRP-14 and huMRP-14 were tested (Figure 4.4, page 142).

In general, the proteins that were used in the *in vitro* systems were endotoxin depleted. This process caused a percentage of Triton X-114 detergent to be left behind. The decision was made early on not to attempt to remove this, as the process is associated with a substantial dilution of protein concentration (the importance of protein concentration will be discussed later). The “trade-off” for non-removal of detergent is evidenced in the typical “bell-shaped” adhesion curve with diminishment of adhesion at higher concentrations (Figure 4.2B, page 139). This was thought to be due to the levels of detergent present in the protein, as this finding could be reproduced if huMRP-14 was compared with “undepleted” human MRP-14 tested at the same concentration (M.Robinson, personal communication). Murine MRP-14 was essentially used “neat” at the top concentration to achieve the concentrations that needed to be tested within the assay.

The concentration of MRP-14 used in various assay systems, such as adhesion and the *in vivo* air pouch model was found to be a limiting factor. In the case of the adhesion assay, a plateau was not generally reached. As the downside of the “bell-shaped” curve at higher MRP-14 concentrations was considered to be due to detergent effects, it was of great importance to generate more concentrated MRP-14 than that
obtained from the standard purification procedure, so that higher MRP-14 concentrations could be evaluated without the confounding detergent effects.

Several attempts were made to concentrate MRP-14. These attempts were based around the Centricon system with a 10kDa cut off, in which buffer is removed from the protein by centrifugation over a fine filter; this thereby concentrates the protein. Using this method, MRP-14 concentrations were increased from 0.4mg/ml to >5mg/ml. Unfortunately, this concentrated MRP-14 was found to be almost inactive in adhesion assays, at all concentrations tested. When this protein was subsequently analysed, it was found to be aggregated, as the average molecular mass assessed by light scattering increased from 28±2 to 72±3kDa (A.Coffier, personal communication). Concentration of MRP-14 by centrifugation was hence abandoned.

In summary, high concentrations of MRP-14 (6μM) were associated with a decrease in cell adhesion, generating a “bell-shaped” adhesion curve. Whether this effect was detergent-mediated or was due to other properties of the protein was not completely decided.

One interesting feature of the adhesion data was the seeming increased potency of huMRP-14, compared to muMRP-14, on murine cells. This difference was seen on all the cell types tested but the reason for this difference is unclear. If muMRP-14 and huMRP-14 are acting on the same receptor, the finding that the non-endogenous huMRP-14 molecule is more potent than the endogenous muMRP-14 molecule is unexpected. It seems unlikely that huMRP-14 is acting on a different receptor than muMRP-14, as it seems to share the functional characteristics that have been compared to date, such as proadhesive activity on various murine cell types and cell recruitment in the air pouch model (data not shown). To date, the identity of the huMRP-14 receptor is unknown, although it is thought not to act by directly binding to Mac-1 but via a pertussis toxin sensitive- G protein coupled receptor (198). It is worth noting that muMRP-14 is unable to stimulate human neutrophils to bind to human fibrinogen (M. Robinson, unpublished data).

One possible mechanism for the differential potency may relate to the dimeric state of MRP-14. MRP-14 was found to exist mainly (70%) in the dimeric form (section 3.5.2, page 124). HuMRP-14 is synthesised in vivo in 2 different forms, full length and a truncated form which lacks the first 4 amino acids. The truncated form does not form a dimer, due to loss of a cysteine residue and this was the form used to
characterise the functions of huMRP-14 (198). A recent report has linked a cysteine oxidation event in muMRP-8 to dimerisation and loss of chemotactic function (91). This was not due to the cysteine oxidation itself but to the dimerisation event, which was hypothesised to decrease accessibility to the chemotactic hinge region. Although dimeric huMRP-14 was active in adhesion assays (R. Newton, unpublished data) it may be that the dimeric muMRP-14 is inactive and that only the monomer mediates the biological effects of muMRP-14. Therefore, it would be of considerable interest to produce monomeric muMRP-14, perhaps by reduction and alkylation (as described by (245)) and evaluate its function in adhesion and other, functional assays.

The most serious flaw with the muMRP-14 adhesion data is the lack of good antibody blocking data. In previous work, huMRP-14 was found to cause the adhesion of human neutrophils to human fibrinogen in a static adhesion assay with dependence on the β2-integrin, Mac-1 (198). Given that muMRP-14 and huMRP-14 also stimulated the adhesion of various murine cell types to human fibrinogen, it was reasonable to initially attempt to block this interaction with anti-Mac-1 antibodies.

Blocking studies in either of the two experimental systems studies, WEHI-265.1 or thioglycollate-elicited, cell binding to fibrinogen were frustrating. On no occasion was >85% blocking seen, with either of the blocking Mac-1 antibodies tested (M1/70 and 5C6). Although 85% blocking sounds reasonable, this should be placed in context with a variability in the blocking of other known Mac-1-fibrinogen adhesion stimulators such as fMLP, which on some occasions was absent when MRP-14 blocking was present. As neutrophils have a large number of Fc receptors and whole antibodies were being used in attempts to block adhesion, the possibility of cell stimulation by the antibodies was considered. To this end, fAb fragments were generated (P. Tessier) and tested in the adhesion blocking experiments. The results obtained were no different to those seen with the whole antibodies, suggesting that cell activation via Fc receptors was not part of the adhesion blocking problem. The involvement of Mac-1 as a mediator of MRP-14 induced adhesion, was not definitively proven.

Other groups appear to have had similar problems with the blocking of Mac-1-fibrinogen mediated events. In one study (56), peritonitis induced cells, from either buffer control or 1μg MRP-8 were pre-incubated, on ice, for 30 minutes, with an isotype control (1μg IgG2b) or Mac-1 blocking (1μg M1/70) antibody. It was found that the Mac-1 antibody caused 40% blocking and the isotype control, 20% blocking, of
control adhesion. This is an effective 20% block, caused by Mac-1 inhibition. This
blocking was not seen when cells were allowed to adhere to BSA.

There are other candidate adhesion molecules that could mediate the MRP-14
induced cellular adhesion interaction to fibrinogen including the β3-integrin related,
leukocyte response integrin (42) and CD11c/CD18 (160). A future investigator may
wish to assess the possible roles of these adhesion molecules using blocking antibodies.

One other adhesion system evaluated was the thioglycollate-elicited cell binding
to bEND-5 endothelial cells. In this system, which was not investigated in great detail,
bEND-5 cells were grown in 96 well plates and allowed to reach confluence. Half the
endothelial cells were then stimulated with TNF and the adherence of BCECF-labelled
cells after fMLP or IL-8 stimulation measured. This system did not provide a
background : stimulated ratio greater than 2, as was generally found for most of the other
adhesion systems tested. However, this system may be worth investigating in the
future as a physiologically relevant adhesion model.

4.5.3 Calcium flux

Neither muMRP-8 (50), nor huMRP-14 caused a calcium flux (198) so the finding that
muMRP-14 did cause calcium flux was something of a surprise. However, there is a
precedent for functional dissimilarity between murine and human MRP homologues;
huMRP-8 is not chemotactic whereas muMRP-8 is the most potent chemoattractant
yet identified (138). muMRP-14 was found, paradoxically, to exhibit cell state
specificity but seemingly, not cell type specificity. That is, muMRP-14 did not affect
recruited cells i.e. thioglycollate cells (neutrophils, macrophages and monocytes) but it
did affect naïve, resting cells such as neutrophils, platelets and various mononuclear
cells.

Initial cuvette experiments suggested that MRP-14 was affecting one or more cell
types in peripheral blood. The fluxes induced by MRP-14 were of an extremely
prolonged nature, not returning to baseline in 15 minutes. This contrasts with fMLP,
which could produce a flux and return to baseline within 3 minutes. This exciting data
led to the initiation of confocal investigations in an attempt to dissect out the responding
cell types. These revealed the cell specificity findings detailed above. Unfortunately,
due to the way the cells were fractionated, further separation of responding mononuclear
cell types could not be performed. It would be of considerable interest to further dissect
out cell populations and test their MRP-14 responsiveness. One hypothesis, for the seeming non-specificity of cell types affected by MRP-14, could be that MRP-14 only appears to affect multiple cell types because the activation of one cell type causes the release of cellular factors that then activate the other cell types. This hypothesis could be tested by analysing pure cell populations.

The effect that MRP-14 seems to have on platelet aggregation, is particularly intriguing. When this was observed, the first questions that arose were of the physiological relevance of intracellular neutrophil proteins in causing platelet aggregation. Secondly, what other factors have a similar effect? The first point will be covered in the general discussion at the end of this section. However, another factor intimately involved in platelet aggregation is the serine protease, thrombin.

An unpleasant possibility arose. Was the thrombin used to cleave the His-tag from MRP-14 being carried through the rest of the purification scheme and onwards into the functional assays? This was indeed the case; section 3.5.3 (page 125) describes the assay for thrombin detection and the removal method. Contamination of MRP-14 samples was found to be approximately 2 NIH U/ml. When MRP-14 was diluted and used in the calcium flux assay at 1μM, the thrombin dose was 0.07 IU, and 0.007 IU when used at 0.1μM. Reference to the literature suggests that investigators routinely use between 1U (82), 0.25U (108), 0.2U (117) or 0.1U (154) to aggregate platelets and 0.1U to induce calcium flux (108). 0.1μM MRP-14, a dose that causes excellent calcium flux, contains at approximately 14 fold less thrombin than that the lowest dose reported to cause aggregation and calcium flux. It has been reported that thrombin-activated platelets can stick to monocytes and neutrophils and stimulate them to increase levels of oxidative burst (192). When 2μM MRP-14 (0.14IU thrombin) was used to stimulate superoxide burst in peripheral leukocytes (plus platelets), no oxidative burst was observed, also suggesting that the MRP-14 contaminating thrombin levels were not sufficient to activate platelets.

Clearly, the relative role of MRP-14 versus thrombin in the calcium flux induced in peripheral cells still remains to be finally evaluated and therefore the data should be considered preliminary until further experiments with thrombin-free MRP-14 can be performed.
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4.5.4 Chemotaxis

MRP-14, in contrast to fMLP and IL-8, was found not to cause chemotaxis in thioglycollate-elicited cells. The closely related analogue, MRP-8, has been found to be active at $10^{-13}$M and is one of the most chemotactic proteins identified (137). Given the MRP-8 data and the chemotactic properties of other members of the S100 family such as bovine S100L ($10^{-10}$M for guinea pig eosinophils) (131), human psoriasin ($10^{-11}$M for human neutrophils and lymphocytes) (119) and most recently, human MRP-6 (also known as EN-RAGE) ($5 \times 10^{-7}$M for human monocytes) (102), the question about whether MRP-14 was chemotactic or not was very important.

MRP-14 was tested across a wide range of concentrations, from $3\mu$M to $3 \times 10^{-20}$M, using thioglycollate elicited cells, and was found not to induce chemotaxis. In contrast to the in vitro chemotaxis data, MRP-14 was found to be chemotactic in vivo. This suggested that MRP-14 was acting via an indirect mechanism. However, recent results in the calcium flux investigations pointed towards a MRP-14 functional dichotomy, in which MRP-14 might specifically target naïve peripheral blood cells, but not cells which had already migrated in response to a stimulus.

Calcium flux is widely recognised as an early marker of cellular activation. Given this supposition, it was therefore possible that MRP-14 was able to directly activate resting but not pre-activated cells. Great effort was made to investigate this hypothesis.

As has been previously mentioned, mice have only 2ml of peripheral blood and only 46% neutrophils (as a percentage of the non-erythroid cells) (Figure 4.1B, page 135). This makes the isolation of numbers of peripheral neutrophils sufficient for standard chemotaxis assays difficult, in terms of animal usage and experimental time. For these reasons, the 24 well Transwell system, that had been successfully employed to evaluate the chemotaxis of thioglycollate-elicited cells was not the model of choice to evaluate peripheral blood cell chemotaxis.

The two systems used to assay the in vitro chemotaxis of peripheral cells were the modified Dunn chamber and the Neuroprobe chamber. The modified Dunn chamber (290) relies on direct observation of cell movement to demonstrate chemotaxis. As described in the results section, this system produced an unacceptable level of variation when cells in suspension were used.

The second system was the Neuroprobe chamber which is essentially a miniaturised Transwell system, except that the well cannot be removed individually.
Unfortunately, no results were produced from this system and the reasons for this failure may have been various. A contributory factor may have been the choice of label used; cell labelling was necessary as there were not enough cells in the assay system to count by FACS. Both BCECF-AM and green cell tracker dye were evaluated in the system but cells labelled with either dye did not migrate, even in response to standard stimuli like fMLP.

This system is popular in industrial science and queries of industrial users revealed that the whole chamber is often analysed in the fluorimeter (Astra-Zeneca, conference communication). Unfortunately, whether the fluorescence measured was limited to the lower chamber and hence cells that had truly migrated or whether the fluorescence represented cells adhering to the membrane was not clear. If the latter case were true, then adhesion and chemotaxis were being measured together.

One particularly interesting set of data using thioglycollate-elicited cells has shown that MRP-8 does not cause calcium flux (50), as was found for MRP-14. However, unlike MRP-14, CP-10 can induce chemotaxis of these cells (137, 138). This may suggest that if MRP-14 is directly chemotactic for peripheral cells, it may be acting through a completely different mechanism than is MRP-8.

Clearly, it is of great importance to discover whether the functional dichotomy of MRP-14 with resting and pre-activated cells extends to chemotaxis. A future investigator may wish to make this issue their highest priority. Suggestions for further work may include the evaluation of other fluorescent dyes and the maturation of bone marrow cells into appreciable numbers of peripheral neutrophils with IL-3 (as described in (295)), so that the Transwell system could be re-used for cells which are in an essentially resting state.

4.5.5 Superoxide generation and cell activation

In general, activation of cells can be measured simply, such as by calcium flux. In neutrophils, cellular activation has another meaning and is generally associated with large external changes e.g. shape change, loss of L-selectin, activation of superoxide generation and granule release. Taken altogether, the study data suggested that MRP-14 caused adhesion, indirect chemotaxis and could activate peripheral blood cells, as measured by calcium flux. This section of the study demonstrated that MRP-14 did not activate either peripheral or pre-activated cells to undergo superoxide burst.
One problem with this study was the relatively small increases in fluorescence that were detected with positive stimuli, such as fMLP. These were approximately 2-fold background levels. It is thought that this is due to the sensitivity of the dye, H$_2$DCFDA, that was used. Other investigators have used dihydrorhodamine and have produced shifts in fluorescence of up to 1000 fold when using stimuli such as PMA (259). Given the functional dichotomy of MRP-14, towards resting and pre-activated cells in the calcium flux assay, it would be of considerable interest to repeat these experiments using a more sensitive detector of superoxide generation, in order to investigate whether the present negative conclusions are correct.

4.5.6 *In vivo* model, the air pouch

*In vitro* profiling of the function of MRP-14 revealed that MRP-14 was proadhesive but not directly chemotactic. When *in vivo* investigations were undertaken, it was a surprise to find that MRP-14 was a specific pro-inflammatory agent. MRP-14 was found to maximally active at a dose of 50μg, with maximal inflammation occurring at 6 hours.

Another group has suggested a role for recombinant MRP-14 in *in vivo* inflammation. 200μg of recombinant MRP-14, coupled to Affi-Gel beads, was found to cause neutrophil accumulation at 7 days and mononuclear cell accumulation at 14 days (281).

Adhesion investigations revealed that huMRP-14 was able to induce the adhesion of murine cells, in a similar fashion to muMRP-14. Preliminary investigations in the air pouch revealed that huMRP-14 also caused a cellular infiltrate. Although a full dose-response titration was not performed, there was approximately equivalent pro-inflammatory activity with a 50μg huMRP-14 dose as that seen with 50μg MRP-14 (data not shown).

Another protein with demonstrated *in vivo*, pro-inflammatory activity is MRP-8 (138). *In vitro*, MRP-8 is chemotactic at $10^{-13}$M (which equates to 10pg/ml). In contrast, *in vivo* cell recruitment in the mouse peritoneum required 25μg of hinge-region peptide (56). This produced approximately 65% neutrophils and 35% mononuclear cells at 6 hours although total cell numbers were less than twice those of control levels.

MRP-14 was a specific pro-inflammatory agent in the air pouch model as the closely related protein, human MRP-6 failed to elicit any response. This contrasts with recent results showing that bovine MRP-6 (EN-RAGE) is a potent *in vivo* pro-
inflammatory agent in mice (102). However, this sort of species difference is not without precedent as murine MRP-8 but not human MRP-8 has been shown to be chemotactic in vivo (138).

One of the major concerns with the study was the possible influence of LPS. As MRP-14 was produced from E.coli, purified protein is laden with LPS. In the case of MRP-14, at a concentration of 540μg/ml, the LPS level was found to be 0.75μg/ml; this would have resulted in a LPS dose of 15ng with a maximally inflammatory, 50μg MRP-14 dose. However, after endotoxin depletion, this level fell to 15pg with a 50μg MRP-14 dose. Preliminary results suggest that 15ng would result in a small pro-inflammatory effect of approximately twice buffer control in the air pouch model; a 15pg dose was not tested but the LPS effect was background at 0.1ng (data not shown). This data suggests that LPS is not mediating the MRP-14 pro-inflammatory response.

Other data suggesting that LPS is not mediating the MRP-14 pro-inflammatory effect is shown in Figure 4.15A (page 172). 1μg LPS was found to mediate a pro-inflammatory response with a maximal response time of 12 hours, versus a maximal response time of 6 hours with MRP-14.

Taken together, these data suggest that the MRP-14 pro-inflammatory effect is not mediated by LPS. However, in order to be completely sure, one solution would be to perform a small air pouch experiment, testing the activity of MRP-14 and LPS in 2 congenic strains of mice, C3H/HeB LPS-sensitive and C3H/HeJ LPS-insensitive mice. The defect causing LPS insensitivity in C3H/HeJ mice has been shown to a mis-sense mutation in the Tlr4 (Toll-like receptor-4 gene) gene causing proline to change to histidine (207). This system has previously been used to assess the influence of LPS on various phenomena (65, 167).

One of the other main questions raised by the results of the in vivo investigation is what is mediating the MRP-14 induced response? The two main options are that MRP-14 is directly chemotactic, a possibility that cannot yet be completely ruled out (see section 4.5.4, page 181) or that MRP-14 causes the release of a factor, or factors that then mediate the subsequent inflammation.

One means of identifying the nature of the stimulant would be to evaluate the type of cellular infiltrate. All stimuli tested, LPS, TNF, MRP-14 and MIP-2 caused approximately 30% macrophage/monocyte and 70% neutrophil infiltrate. Given this homogeneous data, it seems pointless to speculate on the mediator(s) of the MRP-14
response which could be either TNF-α or MIP-2, or both, among many other chemokines.

*In vivo* cell recruitment, in the absence of *in vitro* chemotaxis activity, suggested that MRP-14 was acting indirectly to recruit cells. Circumstantial evidence suggesting that MRP-14 may not be acting directly comes from the time-course of the air pouch infiltrate. MRP-14 was shown to have a maximal recruitment at 6 hours versus 12 hours for LPS and 2 hours for the directly acting chemokine, MIP-2.

Murine MIP-2, as used in the air pouch assays, is a direct acting chemokine and acts by binding the single IL-8 receptor (150). Another mouse chemokine, KC, has been shown to act with MIP-2 to be the predominant direct chemoattractants for murine neutrophils *in vivo* (25). On the other hand, LPS is an indirectly acting agent which generally activates the host immune system and initiates septic shock. LPS has been shown to induce the production of MRP-8 but nor MRP-14 in monocyte / macrophage cell lines (107). LPS has also been shown to cause the transcription of a host of cytokines, including TNF-α, MIP-2, IL-1β and IL-6 after rat lung instillation (275).

The different mechanisms of action of MIP-2 and LPS may explain the different maximal recruitment kinetics in the air pouch. As MRP-14 had a maximal recruitment at 6 hours, this may suggest that *in vivo* it may act directly but in addition may cause the release and / or synthesis of a variety of other chemotactic factors as it is not as fast as a direct chemokine but is faster than a completely indirect factor.

It is also possible that MRP-14 might exert its chemotactic effect through the release of MRP-8. In this scenario, it is possible to envisage the inflammatory reaction being abrogated, over the course of 12 hours by the formation of the MRP-8/14 complex. Given that in humans, the MRP-8/14 complex is functionally inactive, whereas the MRP-14 subunit induces adhesion (198), it is possible that the murine MRP-8/14 complex is also functionally inactive. This would provide a mechanism for the resolution of the inflammatory stimulus. Arguing against this hypothesis is the preliminary finding that MRP-8 KO mice respond more strongly to MRP-14 recombinant protein than MRP-8 WT mice (see section 6.2.9, page 262).

A recent study revealed a wide spectrum of inflammatory activities for bovine MRP-6 (EN-RAGE) including adhesion, chemotaxis and *in vivo* cell recruitment; these effect were mediated through RAGE (102). The anti bovine EN-RAGE antibody recognised a factor in murine plasma that was elicited by LPS; this may be murine...
MRP-6. However, murine MRP-6 has yet to be identified and examination of the EST libraries has failed to find a murine homologue to human MRP-6 (218). This suggests that either murine MRP-6 is too divergent from human MRP-6 to be identified from homology searches or that mice do not have a MRP-6 homologue. If this is the case, then the anti bovine EN-RAGE antibody must have bound a factor in murine plasma with high EN-rage homology. The most homologous protein to EN-RAGE is MRP-14 (173). It would be particularly interesting to discover what factor in murine plasma is recognised by the anti-EN-RAGE antibody and also whether MRP-14 activates cells through RAGE.

This question of MRP-14 signal transduction may be resolved in the future, whether or not MRP-14 is directly chemotactic, with the use of RT-PCR on air pouch material recovered after stimulation with MRP-14.

4.5.7 Comparison of human and murine MRP-14

One of the stated aims of this chapter was to compare the function of human and murine MRP-14. If the in vitro functions of the human and murine proteins had been the same, then this would have allowed speculation as to possible functions for huMRP-14, in vivo, from the function of muMRP-14, in vivo.

Murine and human MRP-14 share functional properties but are not identical. Similar properties include, adhesion, superoxide burst and chemotaxis. muMRP-14 causes adhesion of myeloid cells to fibrinogen, but the adhesion molecule that mediates this effect is not known. In contrast, huMRP-14 causes adhesion to fibrinogen via the β2-integrin, Mac-1 (198). Neither mu- nor huMRP-14 (198) cause superoxide burst or chemotaxis (still under investigation for muMRP-14).

In vivo, muMRP-14 was found to be an indirectly acting pro-inflammatory agent, causing neutrophil and monocyte recruitment into the air pouch model of inflammation. Preliminary data suggests that huMRP-14 acts in a similar fashion to muMRP-14, in the air pouch model (data not shown).

The main dissimilarity between hu- and muMRP-14 is calcium flux. huMRP-14 does not cause calcium flux (198) whereas muMRP-14 causes calcium flux of naïve peripheral blood cells.

In summary, functional differences do exist between hu- and muMRP-14, in vitro. Although preliminary data suggests functional similarity in vivo in the mouse, it
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It is possible that huMRP-14 is not pro-inflammatory in the human. Given the significant *in vitro* differences, it does not seem wise to derive any implications about the function of huMRP-14 *in vivo*, from the muMRP-14 *in vivo* data.

4.5.8 Future work

As has been alluded to in the various discussion sections, there are portions of the project that need more experimental data in order to draw satisfactory conclusions about the function of MRP-14. This would include, satisfactorily blocking the proadhesive activity of MRP-14 with antibodies, evaluating the abilities of MRP-14 to induce (or otherwise) naïve peripheral blood cells to undergo chemotaxis, identifying the true mediators of the *in vivo* pro-inflammatory response and evaluating the response of LPS-insensitive mice to MRP-14.

The question about *in vitro* chemotaxis needs to be evaluated as a priority as the search for other *in vivo* mediators would be less of a priority if MRP-14 was shown to be directly chemotactic for naïve peripheral blood cells.

As a secondary phase of investigation, it would be particularly interesting to test the role of the MRP-14 monomer versus dimer in all the assay systems. As alluded to in section 4.5.2 (page 176), it is possible that the biologically active MRP-14 molecule is a monomer.

Although this investigator has had considerable difficulty in isolating MRP-8 (section 3.3, page 111), if it did become possible to isolate MRP-8, then it would be interesting to first repeat the *in vitro* and *in vivo* chemotaxis results that have been reported (137). Subsequently, it would be interesting to combine purified MRP-8 and MRP-14 to see if they would complex (this has been shown in boiling conditions using His-fused proteins (127)). Given that both proteins appear to be chemotactic *in vivo*, it would be very interesting to know if they could negatively regulate each other. That is, would the complex be inactive *in vivo*?

Also, as cysteine oxidation and dimer formation has recently been shown to be important in the abrogation of the chemotaxis induced by MRP-8 (91), it would be of considerable interest to place MRP-8 and MRP-14 together and examine whether a heterocomplex was formed, associated with cysteine oxidation and whether there was a difference in pro-inflammatory effect between cysteine oxidised and cysteine unoxidised complexes.
4.5.9 Hypotheses about the physiological role of MRP-14

The data accumulated in this study has suggested a role for MRP-14 in recruiting cells from the bloodstream to an inflammatory site. A schematic of possible sites and mechanisms of action modelled on the paradigm of leukocyte rolling in, and extravasation from the vasculature is shown in Figure 4.18 (page 190).

One crucial question is, what could be the source of MRP-14 at an inflammatory site? MRP-14 is an intracellular molecule, found in myeloid cells and lacks a signal sequence. Both MRP-14 and MRP-8 have been found in murine abscesses, both as dimers (which may be non-functional) and as monomers, at incredible concentrations of 2mg/ml monomer (129). It can be imagined that MRP-14 could be present as a functional unit at many other inflammations where neutrophils or monocytes play a role.

In this study, MRP-14 has been shown to have a role in causing the adhesion of cells to fibrinogen, presumably via Mac-1. Mac-1 is a promiscuous receptor. However, a recent report has suggested that Mac-1 is important for sustaining interactions under flow conditions, to ICAM-1, initiated by LFA-1 (195).

It is also possible that MRP-14 mediates its pro-adhesive and in vivo cell recruitment activity via RAGE. RAGE has been recently shown to mediate a host of pro-inflammatory reactions, including adhesion and both in vitro and in vivo chemotaxis through interaction with the MRP-14-related molecule, EN-RAGE (bovine MRP-6) (102). It would be of interest to know which integrins, if any, are activated after RAGE ligation.

With reference to a putative role for MRP-14 in platelet activation, a recent report has suggested a role for activated platelets as bridging molecules in adhesion to endothelium, utilising GPIIbIIIa on the platelet and αvβ3 and ICAM-1 on the endothelium (24). Also, platelet P-selectin can bind neutrophil PSGL-1 and activate Mac-1 via activation of a tyrosine kinase (70). The activated neutrophil Mac-1 can then bind fibrinogen ligated by the platelet integrin GPIIbIIIa and also neutrophil LFA-1 can bind to platelet ICAM-2, increasing the firm adhesion (267). MRP-14 may activate both the platelet and the neutrophil, which bind to each other and act as a bridge between the neutrophil and the endothelium. This may then act to increase the efficiency of cell extravasation from the vasculature. Furthermore, Mac-1 has been
shown to play a role in the transmigration of leukocytes from the vasculature, across
platelets (57).

MRP-14 is also chemotactic, although whether this is indirect or not remains to
be finally decided. MRP-14 may therefore also act to attract cells from the vasculature
to the inflammatory site, either with or without involving other mediators.

In summary, MRP-14 may play a role in firm adhesion, directly and via
platelets. Also, if Mac-1 activation is proven, MRP-14 may be involved in the
transmigration of leukocytes from the vasculature. MRP-14 is involved in the attraction
of leukocytes from the vasculature to the inflammatory site.
Figure 4.18: Possible roles of MRP-14 in vivo. Direct solid lines indicate a proven role in a specific stage of neutrophil transendothelial migration whereas lines leading to question marks refer to unproven or incompletely characterised functions or unknown mediators. Diagram adapted from an original figure within (244).
5. MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

5.1 Introduction

This chapter describes the first parts of the process undertaken in the creation of knockout mice; gene cloning and analysis, construct design and assembly, followed by ES cell transfection and recombinant selection. It also describes the further characterisation of the MRP-14 gene by fluorescent sequencing and subsequent sequence analysis.

The sequence of events involved in construct generation was as follows. Genomic probes were generated by PCR from exonic oligonucleotides and these probes were then used to probe a λ-phage 129 library. Once a positive λ-phage had been identified, this was subcloned into *E. coli*, and mapped using restriction enzymes and Southern hybridisations. These maps were then used to design knockout constructs based on an insertion-replacement strategy.

As was found during cDNA cloning and protein generation, the set of problems encountered during MRP-8 and MRP-14 targeting were individual. Therefore, the targeting of each gene will be described in a separate section although the work was carried out simultaneously.

At the initiation of the MRP-8 and MRP-14 projects, it was decided not to use a gene deletion strategy i.e. not to remove the ATG-containing exon 2 and possibly some intron, with a convenient restriction enzyme. This was to avoid the possibility of losing any possible intronic promoters in a deletion that might influence downstream genes and hence the knockout phenotype.

Various authors have questioned the validity of conventional knockouts (223) with particular reference to the fact that a conventional knockout mouse is an adaptive organism that has developed without the aid of the deleted protein. Therefore, any conclusions drawn from the use of such a system are flawed. Without using a Cre-loxP system, with tissue specific promoters, such as that recently reported for the post-natal hippocampal CA1 knockout of NMDAR1 (261), these concerns cannot be addressed. However, not altering the expression of other genes by possibly removing promoters seemed to be the safest approach and hence was the one taken.
5.2 MRP-8

5.2.1 Probe generation and library screening

This step was performed with the intellectual input of Florian Otto (ICRF, UK). The first step in the project was to generate a probe that could be used to “pull out” MRP-8 from a genomic library. Possible templates for this probe were the cDNA sequence of murine MRP-8 (140); the genomic sequence of MRP-8 was not known at that time. Comparison of the cDNA sequence with the human MRP-8 cDNA (139) and also the human MRP-8 genomic sequence (139), coupled to the information that genomic structure seems to be conserved across species in this gene family (294), allowed a prediction of the probable intron-exon boundaries. This was important so as to prevent the design of primers across such boundaries.

Primers could have been designed to amplify only exon 2 or 3, which may have been successful in locating MRP-8 within the λ-phage library. However, as there was a certain amount of information from the cDNA sequence already available, the decision was taken to produce a large fragment in order to maximise the chances of identifying a genomic clone.

As was discussed in the Introduction, MRP-8 has a genomic structure comprised of 3 exons with the translational start site in exon 2. Exon 1 is very small, so primers were not designed in this area to minimise the chance of producing a spurious product. Rather, primers were designed throughout exons 2 and 3 so as to produce a fragment encompassing exons 2, 3 and the intron in-between.

PCR products were generated from 129 genomic DNA, prepared from liver, with all combinations of oligonucleotides designed. These PCR products revealed the location and size of the exon 2 intron which was found to be 157 base pairs (data not shown), as compared to the analogous human intron which is 150bp (139). The longest PCR product, 400bp, produced from the oligonucleotides 8F1 and 8B2, was cloned into the pAMP-1 vector, by virtue of the uracil containing repeats at the 5’- and 3’- prime termini.

This vector was sequenced (data not shown). This analysis revealed that the PCR product was specific as it contained the expected portions of exons 2 and 3. Further identical PCR reactions were used to generate more product that was purified, labelled and used to probe a λ-phage library. This probe was called MRP-8 F1/B2.
Chapter 5: MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

A λ-phage library was prepared exactly as described in section, 2.14.2.9.2 (page 86) and then used to locate and clone a MRP-8 homologue. NM536 host bacteria were prepared in the presence of 0.2% maltose to induce expression of the lamB gene, contained within the maltose-operon. This gene effectively codes for a λ-phage receptor and increases the rate of infection of bacteria by λ-phage (224). The best plating efficiencies were obtained with fresh NM536 cells. Efficiency deteriorated over time and is the reason for the stipulated 3 week shelf life of the maltose-induced bacteria.

4 MRP-8 hybridising λ-phage were isolated after 3 rounds of 32P-dCTP hybridisation with the MRP-8-F1/B2 probe. These clones, named 8λ1, 8λ2, 8λ3 and 8λ4 were miniprepped and the extracted DNA was used to both confirm the positive identity of the λ-phage and also to begin the mapping process.

Once confirmation was obtained that all 4 clones were MRP-8 positive, (by DIG hybridisation, for 32P-dCTP / DIG differences, see section 2.14.1.12, page 79) 8λ1, 8λ2 and 8λ3 were stored and developed no further. On average, each λ-phage contains between 10 and 15 kb of sequence, which is usually enough to generate a knockout construct, particularly if the gene is compact, as both MRP-8 and MRP-14 are. If it had turned out that the λ-phage clone taken forward for development did not contain sufficient sequence to generate a knockout construct or, was in fact not MRP-8, clones 8λ1, 8λ2, and 8λ3 could have been rescued from storage and subsequently used.

At this point in the project, it was necessary to physically characterise the MRP-8 gene, so that possible strategies for making a knockout construct could be evaluated. To this end, 8λ4 was restricted with a panel of enzymes and subjected to DIG Southern hybridisation with the MRP-8 F1/B2 probe. This produced a very basic understanding of the gross MRP-8 genomic structure, as contained within the λ-phage. Detailed results are presented in Table 5.1 (page 194).

Human data (139) suggests that MRP-8 is a small, tightly clustered gene. Given the human : murine homology, it seemed likely that, as the MRP-8 F1/B2 probe covered exons 2 to 3, and that there were positively hybridised fragments of 8kb, that the entire gene would indeed be contained within this particular λ-phage clone. Working with this hypothesis, it was decided to try and subclone some of the fragments that hybridised to the MRP-8 F1/B2 probe and thereby characterise the gene further.
Chapter 5: MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hybridising Fragment size (kb)</th>
<th>Enzyme</th>
<th>Hybridising Fragment size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI</td>
<td>-</td>
<td>BamHI</td>
<td>4</td>
</tr>
<tr>
<td>BglII</td>
<td>-</td>
<td>Clal</td>
<td>9.5</td>
</tr>
<tr>
<td>EagI</td>
<td>&gt;12kb</td>
<td>EcoRI</td>
<td>3</td>
</tr>
<tr>
<td>EcoRV</td>
<td>3</td>
<td>HindIII</td>
<td>2.5</td>
</tr>
<tr>
<td>Asp718</td>
<td>8</td>
<td>NotI</td>
<td>-</td>
</tr>
<tr>
<td>PstI</td>
<td>3</td>
<td>SacI</td>
<td>8</td>
</tr>
<tr>
<td>KspI</td>
<td>&gt;12</td>
<td>SalI</td>
<td>&gt;12</td>
</tr>
<tr>
<td>SpeI</td>
<td>5.5</td>
<td>Xbal</td>
<td>3</td>
</tr>
<tr>
<td>XhoI</td>
<td>&gt;12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: Summary of preliminary Southern hybridisation mapping of the MRP-8 gene contained within the λ-phage, 8λ4.

5.2.2 Cloning of MRP-8 and gene mapping

The first set of fragments to be cloned were part of various investigations into cloning techniques. The fragments were Xbal digests of 8λ4, shotgun cloned into the common cloning vector, KS+, which was digested with SpeI (sticky ends are identical for SpeI and Xbal although ligation of these ends together allows recleavage by neither enzyme). One positive recombinant, called clone 0, was identified by DIG hybridisation with the MRP-8-F1/B2 probe, suggesting that it carried exons 2 and 3 of MRP-8. There were other recombinants that did not hybridise to this probe; these contained Xbal flanking regions of 0 and were retained for future use.

The next set of fragments to be cloned were EcoRI 8λ4 fragments, shotgunned into KS+. Restriction analysis of these fragments, followed by DIG Southern hybridisation analysis with the MRP-8-F1/B2 probe, identified the EcoRI subclone containing exons 2 and 3. This clone was called, M. Furthermore, analysis also revealed a useful overlap (see Figure 5.1 on page 196) with the XbaI clone, 0.

M was mapped using basic restriction analysis with most of the common cutting enzyme available in the multiple cloning site (MCS) of KS+. More advanced techniques were not required for this mapping as the fragment was only 2.8 kb and most enzymes were, at most, double cutters. Furthermore, at this time, a German group published the sequence of the EcoRI encompassed, coding region of the mouse MRP-8 gene (191),
which is what M was believed to be. The Nacken sequence was computer analysed, generating a full of restriction map of the area; this confirmed the identity of M.

To properly map the gene, rather than just subclones of the gene such as M and O, it was important to know the order and orientation of the various 8λA subclones, in the complete phage and hence, the mouse genome. The overlap between 0 and M defined two new probes. At the 5' end was 8-5'-#1 and at the 3' end, 8-3'-#1. These probes were employed in an attempt to identify the next EcoRI subclones, 3', and XbaI clones, 5', to the ones already identified.

The 3' area identified, was an 8kb EcoRI flanking region called, E. DIG hybridisation of 8λA with the new 5' probe, 8-5'-#1, did not produce an answer; therefore a larger probe, 8-5'-#2, was used. This probe suggested that there was not a large amount of 5' sequence left, and indeed not a whole other XbaI or EcoRI fragment, within 8λA.

The MRP-8 3' flanking region, E was more difficult to map than M or O, due to its larger size, 10.7 kb. The approach taken to map this fragment, was the one taken subsequently for all large fragments in the project, that of restriction followed by DIG Southern hybridisation with plasmid and insert derived probes. An example of the results obtained using this technique is shown in Table 5.2 (on page 197), and the map that this type of analysis produces is shown in Figure 5.2 (on page 196). The conclusions drawn were based on the map of KS+ and its MCS. For example, BamHI cuts once in the MCS, and as 2 fragments of 8.1kb and 1.7kb are produced then there is 1 site in the insert. The 8.1kb fragment hybridises with, and hence contains, KS+ and the 1.7kb fragment contains the insert probe. From analyses such as this, the provisional map of E was drawn.

If the precise localisation of restriction sites was (were) not definitely determined following the first round of digests and hybridisations, then they were subsequently determined with multiple digests using one enzyme for which the site(s) was (were) definitively known plus the enzyme in question. In some cases, evidence was built up in stages with a series of double digests, each revealing the location of 1 site. This fine mapping was important as it facilitated later gene targeting planning. The orientation of clone E in the genome was determined from the position of the probe 8-3'-#1, as this had been derived from O, whose orientation was known.
Figure 5.1: Schematic restriction map of 2 overlapping 84 clones, O and M.

Figure 5.2: Provisional restriction map of E, as determined from Southern hybridisation data obtained after hybridisation with KS+ and 8-3'-#1 probes.

Figure 5.3: A schematic of the way hairpin oligonucleotides may bind to the termini of a linearised DNA molecule and prevent exonuclease digestion. The boxed bases represent a NotI terminus in a linear DNA molecule. Complimentarity and the lack of it between bases is represented by a dotted line and its absence, respectively.
Chapter 5:- MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fragments produced (kb)</th>
<th>8-3'-#1 hybridising</th>
<th>KS⁺ hybridising</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Asp718</td>
<td>3.6, 3.1, 3.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>BamHI</td>
<td>8.1, 1.7</td>
<td>1.7</td>
<td>8.1</td>
</tr>
<tr>
<td>BglII</td>
<td>3.8, 3.1, 1.9, 1.4</td>
<td>1.4</td>
<td>3.8</td>
</tr>
<tr>
<td>ClaI</td>
<td>5.8, 4.2</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>EcoRI</td>
<td>7.5, 2.9</td>
<td>7.5</td>
<td>2.9</td>
</tr>
<tr>
<td>EcoRV</td>
<td>6.9, 3.6</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>HindIII</td>
<td>3.4, 2.7, 2.4, 1.4, 0.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Not I</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>PstI</td>
<td>7.8, 1.8, 0.7</td>
<td>1.8</td>
<td>7.8</td>
</tr>
<tr>
<td>SacI</td>
<td>7.8, 2.2</td>
<td>2.2</td>
<td>7.8</td>
</tr>
<tr>
<td>SpeI</td>
<td>8.7, 0.4</td>
<td>8.7, 0.4</td>
<td>8.7</td>
</tr>
<tr>
<td>XbaI</td>
<td>3.6, 2.7, 2.7, 0.5</td>
<td>0.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 5.2:- Summary of preliminary Southern hybridisation mapping of E.

The final subclone generated from λ4 was again isolated from a shotgun ligation; this time from an Asp718 λ4 digest. The aims of this step were to clone the rest of λ4 that was 5' to M (as Asp 718 was known to be in the MCS of this strain of λ-phage) and if possible, produce one fragment that would contain enough DNA to simply insert a resistance cassette and use directly for homologous recombination. This clone, known as 8J13 contained 7.6 kb of insert and was mapped in an analogous fashion to clone E. The first 2.4kb of clone 8J13, that running from the 5' Asp718 site to the SpeI site was considered to be part of the λ-phage arm and so not considered in planning for the knockout construct. Maps of all the MRP-8 subclones used in MRP-8 knockout construct planning and assembly are shown in Figure 5.4 (on page 198). Other plasmids used in MRP-8 knockout construct generation are shown in Figure 5.5 (on page 199).

Once the MRP-8 subclones had been mapped and analysed, there was almost 13kb of contiguous genomic sequence available from the 5' SpeI site in clone 8J13 through to the 3' EcoRI site in clone E. This was then used as the base from which to plan the gene targeting strategy and hence potential construct assembly.
Figure 5.4: Restriction maps of all plasmids used in MRP-8 knockout construct generation and planning. Each sequence is represented with the 5'-end on the left. Plasmids are shown in coding 5'-to 3'-order as found in the genome i.e. 8J13, M and E. Plasmids 8J13 and M and also plasmids 8J13 and E overlap. Plasmid M is represented within 8J13 (labelled A). The 5'-most portion of E is represented within 8J13 (labelled B).
Figure 5.5: Restriction maps of neomycin resistance-cassette containing plasmids used to generate MRP-8 and MRP-14 neomycin knockout constructs. 203
5.2.3 Neomycin interruption construct generation

As was discussed in some detail in the Introduction, it was decided to knockout MRP-8 and MRP-14 by insertional replacement rather than deletion, to try and avoid altering the function of any other genes, by possible alteration or deletion of intronic promoter or enhancer sequences.

At the outset of the project, a detailed knowledge about the promoter sequences of both MRP-8 and MRP-14 was lacking. Therefore, it was also decided not to use promoterless selection cassettes in the constructs. These cassettes require insertion after a functional, active promoter for activity; they will not confer antibiotic resistance to an ES clone unless they are inserted downstream of an active promoter. Therefore, if the targeted gene promoter lies external to the construct then cells that have undergone a random recombination event will die in neomycin selection, as those cells will not be actively transcribing the Neo resistance gene. In summary, with this strategy, the percentage of correctly targeted antibiotic resistant ES clones screened can be higher than the percentage found when screening cells transfected with a promoter-carrying antibiotic resistance gene.

To decide how to disrupt the MRP-8 gene, the published genomic (191), cDNA (140) and the deduced 8A4-derived sequences were examined. The transcriptional start site for MRP-8 is in exon 2. The first restriction site available in exon 2 for gene disruption with a resistance cassette was a DrdI site (just after the transcriptional start site). This was unsuitable as firstly, DrdI is a relatively common cutter (recognition sequence GACNNNNNNGTC), and secondly, cloning would be difficult as due to the non-specific central region of the recognition sequence, the vector and insert would need to be blunt ended. Therefore it was decided to insert a restriction site immediately after the ATG start codon by mutagenesis. This would have the added benefit of preventing expression of even a truncated peptide. Such a consideration was particularly important given the overall small size of the protein (89aa). Potentially, even a short sequence may be enough to generate a functional moiety.

The MRP-8 restriction map suggested that a rare cutting enzyme site should be introduced to allow cassette introduction, as more common enzymes were present within the construct. A construct-unique XhoI site was chosen to allow insertion of the neomycin cassette from pMC1 (Stratagene). Unfortunately, the neomycin-LacZ cassette, used in the MRP-14 construct, could not be used as BamHI and BglIII sites.
Chapter 5:- MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

were present within the MRP-8 construct (BamHI is the only site available to cut out LacZ-neomycin from its plasmid) and blunt cloning was not a viable option for this investigator.

A graphical representation of the mechanics of MRP-8 neomycin knockout construct generation is shown in Figure 5.6 (on page 202). Plasmid 8J13 (spanning the region from the 5'- Asp718 site to the Asp718 site after the end of exon 3) was digested with BamHI; this caused removal of a BamHI (multiple cloning site {MCS}) to BamHI (before exon 1) fragment, leaving a 2.4kb insert fragment [step 1 in Figure 5.6]. Single stranded DNA was then generated by infection with helper phage and an Xhol site introduced, after the start codon in exon 2. This was achieved by in vitro mutagenesis using the Sculptor kit, the product was plasmid A4 [step 2]. Plasmid A4 was then linearised at the 3' end on Asp718 and a 3.1kb Asp718 fragment from plasmid E (spanning the region from the EcoRI site after the end of exon 3 to the EcoRI site at the end of the characterised DNA), generated by a double digest with EcoRI and Asp718, was subcloned in to produce plasmid 6 [step 3]. Plasmid 6 was linearised in exon 2 by Xhol and a 1.1kb SalI / Xhol Neomycin resistance cassette from pMC1 (Stratagene) was subcloned in reverse transcriptional orientation to generate the MRP-8 knockout construct, shown in detail in Figure 5.7 (on page 203) [step 4].

Restriction map assumptions were checked with various diagnostic digests at every stage of construct production, from 8J13 truncation through to final check of the knockout construct, prior to electroporation of ES cells.

The probes used to determine correct gene targeting were evaluated based on the restriction digests available to demonstrate a RFLP that contained a region external to the construct. Externalisation of the probe versus the construct was important as this prevented the detection of non targeted integrations of the construct, that randomly produced the correct fragment length when hybridised. For MRP-8 the 2 candidates available for ES screening were the 5'- probe A, with an EcoRI genomic digest and the 3'- probe B, with a Scal digest (see Figure 5.8 on page 204). Other candidates were evaluated but these did not produce single bands or specific hybridisations and therefore were discarded. Probe A, with an EcoRI digest was chosen for ES screening as EcoRI was considered to be a more efficient enzyme than Scal. A schematic diagram of how homologous recombination should occur after electroporation of the MRP-8 knockout construct in vivo is shown in Figure 5.8 (on page 204).
Figure 5.6: Schematic representation of the steps involved in MRP-8 neomycin knockout construct generation. Step numbers and the name of the plasmid involved are labelled. Areas crossed out in red are areas lost during cloning.
Figure 5.7: Detailed restriction maps of A. the MRP-8 and B. the MRP-14 neomycin knockout constructs.
Figure 5.8: Targeting the MRP-8 genomic locus.
Chapter 5:- MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

5.2.4 ES cell electroporation and selection

All ES cell work for both the MRP-8 and MRP-14 knockouts, including cell maintenance and transfection, but excluding screening, was done at the Clare Hall ICRF unit under the direction of Ian Rosewell by Ian Rosewell, Mary Ann Jacobs and Stephen Wilson.

Both MRP-8 and MRP-14 knockout constructs were linearised with NotI. Construct linearisation is important to ensure that homologous recombination, a replacement event, rather than an insertion event, the incorporation of the whole construct, including Ks⁺, occurs.

ES cells, growing in neomycin-free media, on neomycin-resistant feeder embryonic fibroblasts were electroporated with the linearised MRP-8 knockout construct. After 24 hours, positive selection was applied and single colonies picked at around 10 days. Positively selected, neomycin-resistant colonies were grown up and duplicated. 1 set of colonies was lysed and the DNA used in a Southern hybridisation screening assay and the other set was frozen down, ready to be thawed when a correctly targeted clone was identified.

After the first transfection of the NotI-linearised MRP-8 construct, 288 neomycin resistant clones were picked. Clones were analysed by EcoRI digestion of purified DNA and ³²P-dCTP Southern hybridisation with probe A (see Figure 5.9A, on page 207). Homozygote wild type (WT) colonies produced one band at 2.8kb whereas targeted, heterozygote WT / mutant (MUT) colonies produced two bands of 2.8kb (WT) and 2.4kb (MUT). This transfection had a successful targeting (homologous recombination) frequency of 1 in 288 (see Figure 5.9A, 5F, on page 207).

The targeted clone (5F) seemed to be a mixed clone i.e. a heterozygote WT/MUT clone mixed with a homozygote WT clone. The WT band was brighter than the MUT band on the autoradiograph, generated by ³²P-dCTP hybridisation with probe A (see Figure 5.9A). The clone was further analysed by ³²P-dCTP hybridisation with probe C (data not shown) which lit up the 2.4kb band, confirming correct targeting but not eliminating the possibility that the clone was mixed. To confirm whether the clone was mixed, the clone was grown up and re-subcloned into 40 subclones. Southern analysis of these clones suggested that the original clone was not mixed as all clones seemed to have the autoradiograph profile of the original 5F clone (Figure 5.9A, 5FD10).
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As 2 clones are required for reliable interpretation of the results of gene targeting experiments, a second transfection was performed. For this transfection, the MRP-8 targeting construct was slightly modified to include "hairpin" oligonucleotides, ligated to the NotI ends of the construct. These oligonucleotides form a NotI terminus with a region of non-complementarity forming an open loop (see Figure 5.3, on page 196). 480 clones were analysed exactly as described above, with probe A (Figure 5.9A, 3C11) and probe C (Figure 5.9B), producing a successful targeting frequency of 1 in 480. As with the first transfection, the positive clone had a weaker 2.4kb MUT band than the 2.8kb WT.

To confirm that a replacement rather than an insertion event had occurred in the targeting of MRP-8, the 3'- targeting of various ES cell clones including 5FD10 and 3C11 was subsequently investigated. This was done using a Scalf digest and hybridisation with probes A and C (probe B was not useful, see discussion). This investigation suggested that 5FD10 was correctly targeted (10.4kb MUT and 9.3kb WT, with probe A and 11kb with probe C, Figure 5.9C, D). However, there was an aberration in the 3C11 targeting (9kb WT/MUT with probe A and 9kb with probe C, Figure 5.9C, D). However, it was still thought that a replacement event had taken place (see discussion).

Clones 5FD10 and 3C11 were subsequently taken forward for development to chimeras (see Chapter 6).
Figure 5.9: Southern hybridisation of ES cells transfected with the MRP-8 neomycin knockout construct.

A. Original identification of clones 5F, 5FD10 and 3C11 by hybridisation of EcoRI digested DNA with probe A.  
B. Confirmation of the identity of 3C11 by hybridisation of EcoRI digested DNA with probe C. 
C. Further investigation of various ES cell clones by hybridisation of Scal digested DNA with probe A. 
D. As C. but with probe C.
5.3 MRP-14

5.3.1 Probe generation and library screening

This step was performed with the intellectual input of Florian Otto (ICRF, UK). The cDNA sequence of murine MRP-14 has also been published (140). As was the case for murine MRP-8, sequence comparison of the MRP-14 cDNA with the human MRP-14 cDNA (139) and also the human MRP-14 genomic sequence (139), coupled to the information that genomic structure seems to be conserved across species in this gene family (294), allowed a prediction of the probable intron-exon boundaries. However, it seemed unlikely that a product could be easily generated that spanned the intron between exon 2 and exon 3 as the human intron was 1.5kb.

MRP-14, like MRP-8 and other S100 family members has a genomic structure comprised of 3 exons with the translational start site in exon 2. Exon 1 is very small; therefore, as was considered for MRP-8, primers were not designed in this area to minimise the chance of producing a spurious product. Primers were designed throughout exons 2 and 3.

PCR products were generated from 129 genomic DNA but only with combinations of oligonucleotides from exon 3. Products were not produced that spanned the putatively large intron between exons 2 and 3. The longest PCR product, of 230bp, was produced from the oligonucleotides 14F2 and 14B2. This was called MRP-14 F2/B2 and was cloned into the pAMP-1 vector and sequenced (data not shown). This analysis revealed that the PCR product was specific as it contained the expected portion of exon 3. Subsequent PCR reactions, using the same conditions, were used to generate more product that, once purified on an agarose gel, was labelled and used as a probe in a λ-phage library.

Only 1 MRP-14 positively hybridising λ-phage clone was isolated after 1 round of $^{32}$P-dCTP and 2 rounds of DIG hybridisation. This clone, named 14λ, was miniprepped and the resultant DNA used to both confirm λ-phage identity and to initiate the mapping process.

The same procedure as was used to map and subclone MRP-8 was followed for MRP-14. MRP-14 λ-phage was restricted with a panel of enzymes and DIG Southern hybridised with the MRP-14 F2/B2 probe. Data from this investigation, summarised in Table 5.3 (on page 209), gave a basic restriction map of the MRP-14 gene and identified some cloning targets for the next phase of the project.
5.3.2 Cloning of MRP-14 and gene mapping

The first subclones of MRP-14 generated from 14λ were XbaI subclones produced from a shotgun ligation into XbaI linearised KS+ plasmid. The recombinant plasmid containing exon 3 was called 14 and was identified after DIG Southern hybridisation with MRP-14 F2/B2 and fluorescent sequencing using the 14F2 and 14B2 primers. Unfortunately, from analysis with the cloning enzyme, XbaI, 14 appeared to be a double shotgunned product, i.e. 2 inserts had been cloned in tandem, into KS+.

The introduction of a novel cloning vector, pZero, which relies on interruption of a lethal gene, for survival of transformed bacteria, in combination with shotgun subcloning and super competent bacteria allowed the entire contents of 14λ to be shotgun subcloned into E.coli, in 1 step. A total of 3 species of EcoRI subclones were found, named JE7-4, JE4-4 and JE7-6. Restriction analysis followed by DIG Southern hybridisation was used to identify JE7-4 as the 3' portion of MRP-14, containing exon 3. JE4-4 was found to be the most 5' region of MRP-14, by fluorescent sequencing. This analysis showed that exon 1 was very close to the 3' BgIII restriction site, illustrated in Figure 5.10 (on page 211). Mapping of this subclone was performed in the same fashion as that performed on all other subclones.

Further fluorescent sequencing identified JE7-6 as the intermediate region, containing exon 2 and also the start of the intron lying between exons 2 and 3. Identification of all the regions of the MRP-14 gene and their relative locations allowed
Chapter 5: MRP-8 and MRP-14 knockout construct generation and ES cell cloning. The planning and initiation of knockout construct generation. Restriction maps of all the plasmids used in this process are shown in Figure 5.10 (on page 211). Other plasmids used in MRP-14 knockout construct generation are shown in Figure 5.5 (on page 199).
Figure 5.10: Restriction maps of all plasmids used in MRP-14 knockout construct generation and planning. Each sequence is represented with the 5'-end on the left. Plasmids are shown in coding 5'- to 3'- order as found in the genome i.e. JE4-4, JE7-6 and JE7-4. Sequences adjoin, but do not overlap.
5.3.3 Neomycin interruption construct generation

As was found with the MRP-8 gene, MRP-14 did not have any convenient restriction sites into which a restriction cassette could be inserted. Ideally, cassette insertion would be directly after the initiation codon to prevent production of any truncated protein. Therefore, it was decided to introduce a construct-unique BamHI site after the initiation codon in exon 2; this would allow cloning in of the LacZ-pMC1 Neo cassette.

A graphical representation of the mechanics of MRP-14 neomycin knockout construct generation is shown in Figure 5.11 (on page 214). Plasmid JE4-4 (spanning the region from the EcoRI site at the 5' end of characterised DNA to the EcoRI site in the middle of exon 2) was shortened to 3kb by removal of an EcoRV (MCS) to EcoRV (before exon 1) fragment [Figure 5.11, step 1]. Single stranded DNA was generated by infection with helper phage and a BamHI site introduced by mutagenesis after the start codon in exon 2 to produce plasmid 23 [step 2]. Plasmid 23 was linearised at the 3' end with EcoRI and a 6.5kb EcoRI fragment from plasmid JE7.4 (spanning the region from the EcoRI site between exons 2/3 and the 3' end of characterised DNA) was subcloned in to produce plasmid 4 [step 3]. Plasmid 4 was shortened by removal of a 3.5kb Sacl (at 3' end and in the MCS) fragment to generate plasmid 10 [step 4]. Plasmid 10 was linearised on EcoRI and at 0.3kb exon 2 fragment was inserted to generate plasmid 8 [step 5]. Correct orientation was confirmed by sequencing with the primers 4.4F(50-71) and 7.6B 185-164. Finally, plasmid 8 was linearised in exon 2 with BamHI and a 5.2kb LacZ-Neomycin resistance cassette was ligated in to generate the MRP-14 knockout construct shown in Figure 5.7 (on page 203) [step 6].

Restriction map assumptions were checked at every stage of construct production from JE4-4 truncation through to a final check of the knockout construct prior to electroporation of ES cells, with various diagnostic digests.

The probes used to determine correct gene targeting were evaluated based on the restriction digests available to demonstrate a RFLP that contained a region external to the construct. For MRP-14 the 2 candidates available for ES screening were the 5’-probe D with an XbaI digest and the 3’- probe E, with an BamHI digest (see Figure 5.12 on page 215). Probe E was chosen for ES screening as BamHI was considered a more efficient enzyme than XbaI.
5.3.4 ES cell electroporation and selection

The first transfection of the NotI-linearised MRP-14 construct generated many neomycin resistant ES cell clones of which 384 were picked. Clones were analysed for correct targeting by BamHI digestion of purified DNA and $^{32}$P-dCTP Southern hybridisation with probe E. Homozygote WT colonies produced a single autoradiograph band at 11kb whereas heterozygote WT/MUT colonies produced 2 bands of 11kb and 8kb (see Figure 5.13A on page 216). This transfection produced a successful targeting frequency of 9 in 384.

Only 7 of the 9 WT/MUT clones had equally intense WT and MUT bands on the autoradiograph. 3 clones, 1C, 8C and 12H, with equally intense WT/MUT bands were selected for growth and further analysis with probes C, D and E. Clones 8C and 12H were found to be correctly targeted after probe D (WT 5.5kb and MUT 10.5kb, Figure 5.13C) and probe C (10.5kb MUT, Figure 5.13E) $^{32}$P-dCTP hybridisations of Xba I digests; and probe E (4.7kb WT and 10kb MUT BglII digest, 8kb WT and 11kb MUT, BamHI digest, Figure 5.13B) and probe C (10kb MUT BglII digest and 11kb MUT BamHI digest, Figure 5.13D) $^{32}$P-dCTP hybridisations of BamHI and BglII digests. Clone 1C was not found to be correctly targeted (see BglII and XbaI digested DNA hybridisations with probes C, D and E, Figure 5.13D, C and B, respectively).

A second transfection was performed 18 months later. This also produced numerous resistant ES clones; of 196 resistant colonies picked, 13 were found to be correctly targeted. Of these 13 clones, 7 were investigated (see Figure 5.14, on page 217). All clones except 2H3 and 2H7 appeared to be correctly targeted from Southern hybridisation with probes D and E.

From the first transfection, clones 12H and 8C were taken forward for development, clones 2E5 and 2G1 were taken forward for development from the second transfection.
Figure 5.11: Schematic representation of the steps involved in MRP-14 neomycin knockout construct generation. Steps and the name of the plasmid involved are numerically labelled. Areas crossed out are lost during cloning.
Figure 5.12: Targeting the MRP-14 genomic locus.
Figure 5.13: Southern hybridisation of ES cells transfected with the MRP-14 neomycin knockout construct. A. Original identification of targeted clones by hybridisation of BamHI digested DNA with probe E. B. Confirmation of the targeting of 1C, 8C and 12H targeting by hybridisation of BamHI and BglII digested DNA with probe E. C. Confirmation of the targeting of 1C, 8C and 12H targeting by hybridisation of XbaI digested DNA with probe D. D. As B. but with probe C. E. As C, but with probe E.
Figure 5.14: Southern hybridisation of ES cells transfected with the MRP-14 neomycin knockout construct.

A. Original identification of targeted clones 2G1 and 2E5 by hybridisation of BamHI digested DNA with probe E.

B. Confirmation of the targeting of various clones' targeting by hybridisation of BamHI digested DNA with probe E.

C. As B, but with a BglIII digest.

D. As B, but with an Xbal digest.

E. Confirmation of the targeting of various clones' targeting by hybridisation of BamHI digested DNA with probe D.

F. As E but with a Xbal digest.
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5.3.5 Gene sequencing and subsequent analysis

Whilst gene targeting was underway and the results unknown, two obvious outcomes were possible. Firstly, the knockout mice could be viable and the experiment could proceed; secondly, the knockout mice could be lethal and the experiment would reveal the genes were important somewhere in development and nothing about functions in the adult animal. If the former situation applied then things were ideal but if the latter applied then the laboratory may have wished to consider another targeting strategy, such as Cre-LoxP.

An inducible knockout strategy is ideal for studying the effect of genes important in development as it allows both temporal and tissue specific gene deletion. However, if during the creation of ‘floxed’ genes, the gene promoter or an enhancer element is interrupted then the assumed WT animal may have protein expression that is abrogated to levels that cause morbidity or even mortality. Clearly, given the investment of animals, money and time, it is important to target the gene appropriately. To this end, it was decided to sequence the MRP-14 gene and try to identify the promoter, any enhancer elements and also rare restriction sites that may help in the insertion of LoxP and resistance cassettes. This was not necessary for MRP-8, as the genomic sequence had already been published (191).

Three subclones of the 14λ phage encompassed the whole gene, from exons 1 to 3. These clones were, from 5'- to 3'-, JE4-4, JE7-6 and JE7-4 (as already described in Figure 5.10, page 211). Both JE4-4 and JE7-4 were truncated, approximately 2kb upstream and downstream of the coding gene sequence, respectively. This was done to decrease the amount of work required; the portions closest to the coding gene sequence were retained. Specifically, JE4-4 was digested with EcoRV and 2.3kb from the 5'-EcoRV site to the 3'-EcoRI site retained; a 4kb portion of JE7-4 was cut out with EcoRI (5'-) and BgIII (3'-) and subcloned into a new vector; JE7-6 was not altered (for restriction maps, see Figure 5.10 on page 211).

JE4-4 was originally sequenced using the vector primers T7 and SP6. From the resultant data, new primers were designed, in both 5'- to 3'- sense and antisense (effectively 3'- to 5'- on the sense strand) directions approximately 100bp in from the 3'-most end of the data so that the new data would overlap with the old. Sequences were assembled using the ABI programme, AutoAssembler. In this fashion the whole of the fragment was sequenced and the data eventually assembled into one contiguous
sequence (see Figure 5.15 on page 221). Any gaps that existed that prevented the sequence being read in each the sense and antisense strands were addressed at this point. The identity of the sense and antisense strands were confirmed by the sequence of exons 1 and 2. Borders between vector and insert sequences were identified by assembling vector termini with the appropriate enzyme ends into the assemblages.

As JE7-6 only contained approximately 300bp, sequencing was a simple matter of using the vector specific primers T7 and SP6 (see Figure 5.15 on page 221). Sense and antisense strand identity was simply revealed, as JE7-6 contained half of exon 2 (from the 5'- most region of exon 2 to the EcoRI site at the 3'- most end of JE7-6).

JE7-4 was much more difficult to sequence than either of the other 2 clones. Sequencing was attempted from 3 directions; 5'- using T3, 3'- using T7 and also centrally using the genomic-probe-producing primers, 14F2 and 14B2. (see Figure 5.16 on page 222 ). The main problems in the sequencing of this fragment were data with ambiguity percentages >3% (ambiguities are bases which the computer cannot guess accurately) which prevented the 3'- and 14F2/B2 regions assembling and also the repeated region in the exon 2/3 intron. Once the unclean data (ambiguity percentages >3%) had been discarded the first problem was addressed. The second problem meant that the repeat region was only sequenced 5'- to 3'- on the sense, but not antisense strand. Various attempts were made to sequence this repeat region including cutting out the region of interest to use as a template and using different polymerases that are reported to be more efficient for sequencing repeats; these attempts were not successful.

Finally, all the data were assembled into 1 contiguous sequence of 6579 bases. The data did not overlap at the clone junctions as the clones were adjoining sequences, for example, JE4-4 did not overlap with JE7-6. This was not an issue as the identification of the exons from the cDNA sequences and the knowledge that the clones originated from a contiguous genomic sequence in λ-phage allowed the orientation of the data.

Once the data was assembled, John Sgorous (Bioinformatics, ICRF) analysed the data with various internet based tools (see section 2.17.3, on page 99) and identified the repeat regions and putative promoter elements. Two likely promoter regions were identified, from bases 1257 to 1307 with a confidence of 0.98 and from bases 1823 to 1873 with a confidence of 1.00. The second promoter region is particularly interesting as it overlaps with the first 11 bases of exon 1. The summation of this data is
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represented in the sequence (Figure 5.17, on page 225), and also shown in Table 5.4 (on page 220). This data should provide a means to target MRP-14 using the Lox-P system.

<table>
<thead>
<tr>
<th>Start position</th>
<th>Finish position</th>
<th>Name of repeat</th>
<th>Repeat Family</th>
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<td>327</td>
<td>Lx</td>
<td>LINE/L1</td>
</tr>
<tr>
<td>293</td>
<td>665</td>
<td>L1_MM</td>
<td>LINE/L1</td>
</tr>
<tr>
<td>670</td>
<td>690</td>
<td>(GAAA)n</td>
<td>Simple repeat</td>
</tr>
<tr>
<td>691</td>
<td>840</td>
<td>L1MA4</td>
<td>LINE/L1</td>
</tr>
<tr>
<td>2735</td>
<td>2744</td>
<td>(GAAA)n</td>
<td>Simple repeat</td>
</tr>
<tr>
<td>2745</td>
<td>2864</td>
<td>(GAAA)n</td>
<td>Simple repeat</td>
</tr>
<tr>
<td>3002</td>
<td>3151</td>
<td>B1_MM</td>
<td>SINE/Alu</td>
</tr>
<tr>
<td>3152</td>
<td>3182</td>
<td>AT_rich</td>
<td>Low complexity</td>
</tr>
<tr>
<td>5280</td>
<td>5409</td>
<td>(TC)n</td>
<td>Simple repeat</td>
</tr>
<tr>
<td>5409</td>
<td>5435</td>
<td>(CA)n</td>
<td>Simple repeat</td>
</tr>
</tbody>
</table>

Table 5.4: Summary of the types and locations of the repeat regions identified in the MRP-14 genomic sequence.

<table>
<thead>
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<th>Start</th>
<th>Finish</th>
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<td>386</td>
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<tr>
<td></td>
<td>2</td>
<td>580</td>
<td>2807</td>
<td>2227</td>
</tr>
<tr>
<td>Mouse</td>
<td>1</td>
<td>1887</td>
<td>2263</td>
<td>376</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2424</td>
<td>4628</td>
<td>1956</td>
</tr>
</tbody>
</table>

Table 5.5: Comparison of the intron sizes between exons 1 and 2 (intron 1) and exons 2 and 3 (intron 2) in humans (139) and mice.

This work was partly done by Rachel McMullan, a summer student that this investigator supervised. Her work encompasses the subcloning of JE7-4 and JE4-4 and approximately 50% of the sequences used to assemble the final data.
Figure 5.15: Summary of the sequences used to sequence A. 4.4 and B. 7.6. Sequences are represented conventionally with the 5' end on the left of the diagram. The direction of the arrow indicates which strand is being sequenced (left to right is sense strand sequencing and right to left is antisense strand sequencing) and the label refers to the primer used.
Figure 5.16: Summary of the sequences used to sequence 7.4. Sequences are represented conventionally with the 5'-end on the left of the diagram. The direction of the arrow indicates which strand is being sequenced (left to right is sense strand sequencing and right to left is antisense strand sequencing) and the label refers to the primer used.
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2821  gaagaagag aagagaagaga agaagaaggag gaggagagg gagaagataga aatgtagatagat
gtagatagag ttagata gtagatagat atagatagat atagatagat atagatagat
2941  gtagacaga gagaagataag aataacagc cagataaa aataataaat agatagtgg
3001  aagcagccag ctgtgcagca cacttttaaat cccagctactgt ggaggacgag aagagacag
3061  ttttgtcatcgc ctgtgtcgca gcgtgatggtc gagatgacgt cggagacatc
3121  aagagaccc tgtcgtgaga aataaaaaa attaataattt aaataaataaa
3181  tagatagtag gtagaggag aagagagac agagaaaaag cctttgaggg agtagaatctct
3241  taaatctcag caaattctctgtataagact caggggatc ttaaataaat gcagagatcag
3301  gttcgataggt ttcgtgagag aagaggttaa aacccactc cttcaagat gggcttattt
3361  acaatagaga tcataagacag gtagagggcc atagtcacag ccattttatt aagacatcttt
3421  atatcattag tcggacacttt aagaaagata cgaatgaagtg aagagatagcgc
3481  accatgacca accatgagag ggacaaactaa aagagaggg aggagttgtct gttgtgtggtt
3541  gtcaagatca gtagagtacct ttttttttttaa taacttttccc gcagacagat gttcataataa
3601  tggtgtagcct ctgtggtcct gcctgtccgc caacttgggga ccaagagaaaa ctagagattt
3661  gtctacattg ggattttag ggccagagat atctctttaag tcagacatc agaacagaa
3721  gataactgat cagggttaacc aagaggttca cttgtagctg aatttgagaag attctctctct
3781  gtgcacactc cccagtgggtt ttacctc ttcattgacata ttcacccaca caccagagaa
3841  caggaacctc acacagtaag ttcacagagta cttggaaggg ggtagctgctt
gttcgagggg tcccgacagcg ctaggctgctg atgtagatct cgcagcaccac
3901  acctcgcttc ctgtagtcctc tctagtccctc tataactcag tgcacccctct tggagttcag
3961  cagagaaaaa taggtcttgc ccccttttaaga ccccggtctg ctgtgtttctct gttgtgctc
4021  tccacataag tagactggac aacgtttaac aatcgctgcc ccccgagaaa cagctgggg
4081  ttggagtatg caagtaagag tagccaagag aaaaatctct ccaagagaga
4141  aagagaaaaa aacagccccaa caaacgtgtgt aatgctgtgc ccacaaatc aacacagattt
4201  atatccttc cttccctcctc ccaggccgca cgcacccataa aatagtccttc cccctttagaa
4261  gttgttcccc tttttctttt ttaaaatttataaatgattaa aacaattgtg attctctatat
4321  ttaatttaag cctggtccttt gaaagctgac gagctcagct cgcccgccag aggctctcctt
4381  gagagaacaa gtaggggtct ttagcaagtaa ttagctcatg gacgcactcg
4441  tatactggat ttcacatcct tttttctagct gttccctgct ggtcgactgg
4501  tctgttacctcttct tattttcaaa attcacccctc tggaggatgct ctttttcgct tttttgtctat
4561  ctagacacc gcctgtcctc aggtctgctct cttctatgcccc taacagcctc ctttatattcao
4621  cccacagaaaa gagagaagaga agtagagccct ctaaaagcag atctagggag aocctggaac
4681  aacacagagc aatcagcagga ctgtgtgagga gttgtagctt cgtgatggtct gcgtatagctt
4741  tgcgcgtctct gagaacaggg cccagcgtgg agttggccga cgtgacccccat cgtgacccgg
4801  aggtgtgctgg aagattatcaag ggatgagccc atgtgagccgg cgtcagccccac agttgtaag
4861  gaagagtgtga ctttaatgggcc ttggttctcg gaattaacgaa gataaatatc aataagtttct
4921  ttagcttcctc cattgtcttt ctgctgcttttc ttgacacctc ctgacagagta cctcctttttt
4981  ctctggtctg cttctgcttttc cttggtttttct cttggtttttct cttggtttttct
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5101  cttggtttttct cttggtttttct cttggtttttct cttggtttttct cttggtttttct
5161  gcataagtgt cctctgtcag tgcagagagc aaaaaactgct ttgcctctcc ccagatccccccc
5221  agatctctcct cttacatcctc atgggtatgtt cttatatccag cttatggcct cttttttctt
5281  cttctctttct cttctctttct cttctctttct cttctctttct cttctctttct
5341  cttctctttct cttctctttct cttctctttct cttctctttct cttctctttct
5401  cttctctttct cttctctttct cttctctttct cttctctttct cttctctttct
5461  cttctctttct cttctctttct cttctctttct cttctctttct cttctctttct
5521  tactagtgg agatcaagaa cctctaactct cttggtatgca agagagcag
5581  cattctctcc cccacacatat gatacttaaa tgggtgtgctg tgggtgtgctg

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5.4 Strategies to target both alleles of MRP-8 and MRP-14

5.4.1 Hygromycin interruption construct generation

Before information was obtained about the viability of MRP-8 and MRP-14 knockout mice, it was considered prudent to contemplate the possibility of embryonic lethality. If that had been the case then various steps to continue the project could have included mapping the time and cause of lethality. A potentially more revealing approach would have been to generate an inducible knockout. However, such a project probably would have required more experimental time than was available. An easier approach to generate information about gene function, although not as revealing as an inducible knockout, would be to generate homozygous mutant (knockout) ES cells and then make chimeras. Examination of these animals might generate information about the role of the proteins in adult animals.

There are two approaches that can be taken to generate knockout ES cells from heterozygous cells. The first is to grow the heterozygotes in increasing levels of selection agent (188). Eventually, a conversion to homozygosity (knockout) occurs, in
response to the positive evolutionary pressure supplied by the increased selection. This is screened for in the same fashion as the originally transfected ES cells were screened. This phenomenon has been investigated, and the cassettes that favour this conversion identified. It has been shown that neomycin resistance cassettes (NRC) that carry a mutation, eliminating an XhoII site, are susceptible to this conversion whilst wild-type NRCs are not susceptible (284). When this approach was being considered, it was found that the MRP-8 NRC was mutant whereas the MRP-14 NRC was wild-type. This meant that this approach would be appropriate for MRP-8, but that a second approach must be taken with MRP-14.

The second approach that can be taken to generate knockout ES cells, is to target the second allele of the gene with a second knockout construct, carrying a different antibiotic resistance cassette. Resistance to a second antibiotic resistance is necessary so that if the mutant allele is targeted and hence the original targeted allele lost, that the cell does not survive and contaminate any possible double-negative cells. One choice of antibiotic resistance cassette is the hygromycin cassette. This cassette had BglII sites which allowed cassette excision and non-reversible ligation into the BamHI site of the MRP-14 knockout construct. A linker would be necessary for use with the MRP-8 knockout construct.

One of the features of the MRP-14-Neo construct was the LacZ cassette which would hopefully allow facile expression analysis. Therefore it was desirable to have it in the second, hygromycin construct. Two LacZ genes may mean twice as much LacZ RNA and protein as a single gene. Therefore, it was decided to engineer a plasmid that would contain both the LacZ and hygromycin cassettes for use in interruption strategies.

This was achieved by removing the LacZ cassette from a LacZ-Neo cassette and combining it with a hygromycin resistance cassette to form a new cassette. A schematic diagram of the cloning strategy to achieve this is shown in Figure 5.18 (on page 228). Specifically, the MCS of KS* was excised and a new, oligonucleotide-based MCS was ligated in [Figure 5.18, step 1]. Secondly, the plasmid was linearised, and the IRES-LacZ cassette was excised from LacZ-Neo with a XbaI/SacII double digest and the fragments ligated together [step 2]. Finally, the hygromycin cassette was excised and the construct linearised with BglIII. Ligation generated 2 plasmids with the hygromycin cassette in both orientations [step 3]. The new plasmid, #35, also had MCS sites.
allowing cloning into the MRP-8 (XhoI) and MRP-14 (BamHI) knockout constructs [step 4]. Detailed restriction maps of the new MRP-8 and MRP-14 hygromycin knockout constructs are shown in Figure 5.19 (on page 229).

MRP-8 and MRP-14 hygromycin knockout constructs were linearised with NotI, although they were not hairpin-capped. As events unfolded, it was not necessary to transfect ES cells with either construct; for further discussion of this, see sections 6.2.2 and 6.3.2 on pages 245 and 267, respectively. The effort expended in generating the hygromycin constructs was not altogether wasted, as this entailed generating a new hygromycin cassette which also contained the LacZ expression cassette. This should be a useful resource for future researchers.
Figure 5.18: Schematic representation of the steps involved in LacZ-hygromycin resistance cassette generation. Steps and the name of the plasmid involved are numerically labelled. Areas crossed out are areas lost during cloning.
Figure 5.19: Detailed restriction maps of A. the LacZ-Hyg plasmid, B. the MRP-8 and C. the MRP-14 LacZ-hygromycin knockout constructs.
5.4.2 Increased selection pressure with neomycin

As described in the preceding section, one method to create −/− ES cells from +/- ES cells is to select the cells with high levels of neomycin (188). Using this method, a conversion rate of approximately 50% has been achieved. When the mechanism of heterozygous to homozygous conversion was investigated, it was found to rely on the presence of a neomycin resistance cassette (encodes neomycin phosphotransferase II) containing a specific mutation. This mutation was associated with a polymorphism in XhoII restriction; normal cassettes had a diagnostic 248bp band whereas the mutant cassette had a 638 bp band instead (284). This mutation corresponded to a glutamic to aspartic acid conversion at residue 182, substantially reducing activity; cells with the mutation are less resistant to antibiotics than those with the normal gene. When the cassettes used to create the MRP-8 and MRP-14 knockout constructs were excised and analysed, the MRP-14 cassette was found to be normal whereas the MRP-8 cassette was found to be mutant; that is the MRP-8 cassette could be used to undergo high selection and conversion (Figure 5.20A, on page 231).

Based on that data, ES cells from the 5FD10 clone were grown up under normal levels of neomycin selection and subsequently dispersed to single clones. Single cell clones were then re-grown using high levels of neomycin selection, to induce the heterozygous to homozygous conversion and surviving cells were analysed by Southern hybridisation with probe A.

Although a number of cells survived and 83 clones were tested, none had undergone conversion to homozygosity and all showed the banding typical of heterozygous cells (Figure 5.20B, on page 231). Given that the expected conversion rate should have yielded approximately 40 homozygous clones, it was decided that if knockout ES cells were required then a second targeting event would be attempted.
Figure 5.20: A. XhoII restriction analysis of the cassettes used to generate the MRP-8 and MRP-14 knockout constructs. Normal cassettes carry a 246bp band whereas mutant cassettes carry a 638bp band. B. Southern hybridisation analysis of highly neomycin resistant clones of the MRP-8 heterozygous ES cell line 5FD10. An example of 3 clones is shown, each of which has a 2.8kb and 2.4kb band, indicative of heterozygosity at the MRP-8 locus.
5.5 Discussion

5.5.1 PCR

One negative feature of the excellent pAMP-1 PCR cloning system was insert generation, with its requirement for uracil-containing primers. It was found that Vent DNA polymerase would not produce a product in a reaction with uracil-containing primers. This is thought to be due to the proof-reading capacity of the Vent polymerase; it is possible that the enzyme becomes ‘stuck’ on the non-DNA base and does not polymerise the rest of the strand. It was found that the only way to get a PCR product using the pAMP-requisite, uracil containing primers, was to use Taq DNA polymerase. This has the disadvantage of decreased product fidelity as Taq produces larger numbers of errors than Vent. It has been shown that the average error rates (mutation frequency/bp/duplication) for Vent polymerase are $2.8 \times 10^{-6}$, and for Taq polymerase are $8.0 \times 10^{-6}$ (45).

5.5.2 Cloning

During the cloning of MRP-8 and MRP-14, gene fragment subcloning was something of a sticking point. Various attempts were made to subclone gene fragments, but these were unsuccessful. Previous cloning steps, like those used to clone the MRP-8 and MRP-14 λ-phage probes, had used steps which had extremely high levels of selection built into them e.g. pAMP-1 system. This stage was slightly different, in that cloning any same ended fragments into a single-enzyme linearised vector produces very high levels of recircularisation, the empty vector phenomenon. Furthermore, the fragment sizes involved here were substantial (on average, 3kb or larger) which meant that ligation of insert to vector, rather than vector to vector events were infrequent. Attempts to minimise cloning background with methods such as vector dephosphorylation are associated with a general decrease in ligation efficiency but a particular decrease is seen in empty vector recircularisation (224). However, use of vector dephosphorylation by this investigator resulted in no colony production at all. One explanation of these results might be that any positive recombination events simply were not being picked up as the bacteria were not competent enough.

Another potential problem with the cloning of both MRP-8 and MRP-14 (which were underway concurrently) was the relative “quality“ of the insert DNA, the λ-phage DNA. Examination of the agarose gels revealed good DNA banding with some associated
smearing in the tracks (data not shown). It is possible that this smearing was protein contamination which may have interfered with the cloning steps. A method modification was discovered from the Owen laboratory (P. Ayton, personal communication), which was a modification of the original. In this method λ-phage DNA was released from the phage capsule by the addition of EDTA, before, rather than after, the protein removal, proteinase K digestion step. It seems reasonable that this alteration in methodology would result in the avoidance of a considerable amount of protein. Indeed, observation of DNA, purified using this method, on an agarose gel, revealed it to be free of associated smearing. This DNA preparation method was then used to generate DNA which was then used to successfully subclone the MRP-8 and MRP-14 genes.

It was also decided to purchase super competent bacteria in order to pick up rare events, such as those produced when cloning relatively large DNA fragments. The simultaneous change in λ-phage production methods and the use of supercompetent bacteria were associated with MRP-8 and later, MRP-14, subcloning success.

The subcloning of MRP-14 took somewhat longer than that of MRP-8. Various problems were encountered, which were not just simple cloning failure problems. For example, shotgun cloning of MRP-14 resulted in subcloning of more than one fragment. Eventually, MRP-14 was shotgun subcloned using the supercompetent E.coli and the zero background, p-Zero system, which relies on the interruption of the lethal gene, ccdb, by insert DNA to allow the survival of transformed bacteria. This is analogous to the older, LacZ interruption, blue wide selection method contained in KS^5.

5.5.3 Hybridisation systems

During the course of knockout construct and targeted ES cell generation, two hybridisation systems were evaluated and utilised, the ^32^P-dCTP and the DIG systems. The main advantages of the DIG system over the ^32^P-dCTP system were increased speed, decreased radiation exposure and facile probe re-use (over many months), all of which are important features in a hybridisation-rich project. The DIG system was successfully used to clone the MRP-14 λ-phage, to clone flanking regions of the genes and also to restriction map the genes. Each hybridisation would typically take 24 hours from hybridisation to resultant X-ray film results, rather than 36 hours with the ^32^P-dCTP system (plus a "hot" blot, if cloned DNA was being mapped).

Unfortunately, after much effort, it was found that the DIG system was
ineffective when trying to hybridise to genomic DNA. Therefore, it could not be used from the stage of ES cell targeting onwards. The same probes that had been tried in the DIG system were functional in the $^{32}$P-dCTP system, when probing genomic DNA. Therefore, the switch was made back to $^{32}$P-dCTP for the remainder of the investigation.

In summary, the DIG system is an excellent system with advantages in speed and ease and safety in handling, as long as cloned and not genomic DNA is used in the investigation.

5.5.4 Construct generation

During the planning of the strategy to be taken in generating MRP-8 knockout constructs, it was decided that introduction of a rare cutting enzyme was required for the final step, that of resistance cassette insertion.

On reflection, it would have been possible to use the more useful LacZ-Neo cassette, which has BamHI termini, by the use of short linker oligonucleotides. This would have been advantageous as the cassette removes the necessity for antibodies in expression detection; MRP-8 antibodies have been a significant stumbling block (see chapter 6). If the mutagenesis had been carried out as planned and the LacZ-Neo cassette removed, ready for ligation with a BamHI digestion, linkers could have been designed that would anneal to the BamHI ends and turn them into XhoI ends, allowing sticky end ligation. This is something that was simply not considered. Selection of a recombination event could still be performed in the same way, with the same probes, as there is a convenient EcoRI site. The only potential problem with this strategy would be the potential recombination frequency. As was noted, MRP-8 had a much lower recombination frequency than MRP-14. One hypothesis for this difference could be the length of the 5'- and 3'- homology regions. If insertion of a 5.5kb LacZ-Neo cassette rather than a 1.1kb Neo cassette was attempted, the frequency might be further reduced, to levels which are almost unworkable.

The transcriptional orientation of the resistance cassette in the MRP-8 construct was reversed as a previous report had suggested that the frequency of correct targeting events was increased up to 10 times when resistance cassettes were orientated this way (292).

The choice of restriction site to introduce in the MRP-14 gene by mutagenesis was decided as follows. The first restriction site available in exon 2 (first protein coding
Chapter 5:- MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

exon) for gene disruption with a resistance cassette, according to the published cDNA sequence (140) was a TaqI site (7 codons from ATG). It was decided not to risk production of a truncated protein by knockout animals and to insert a convenient site just after the ATG start codon by mutagenesis. The fortuitous restriction map of the gene suggested BamHI as the 5’ BamHI site was outside the intended construct and the 3’ BamHI site outside of the region of cloned phage. This then allowed the introduction of a neomycin cassette containing the Lac Z gene rather than the standard neomycin cassette used in the MRP-8 construct. It was hoped, that the use of the LacZ cassette would allow specific mapping of MRP-14 expression in the adult and developing embryo.

5.5 Gene targeting in ES cells

An unusual observation with both transfections of ES cells with the MRP-8 knockout construct was a differential intensity in the WT and MUT bands. The reason for differences in intensity of WT/MUT bands in gene targeting experiments is not clear. It is expected that if there is one copy of each allele (WT/MUT) and the bands are similar in size (2.8kb and 2.4kb in the case of MRP-8) then band intensity should be identical. It has been suggested that these differences may be due to the presence of excess feeder cells in each clone, contributing to the WT band (I.Rosewell, personal communication). However, a recent gene targeting report showed a knockout with similar differences in WT/MUT band intensities in both ES cells and heterozygous mice (166) although no explanation was given for these differences.

One interesting feature of the MRP-8 and MRP-14 gene targeting experiments was the differential frequency of accurate targeting. It is unclear why the MRP-14 construct should produce such high targeting frequencies of 1:43 and 1:15 compared with 1:384 and 1:480 produced from the MRP-8 construct. It might be hypothesised that as the total length of homology is the same in each construct (approximately 5.5kb) but the short arm of the MRP-14 construct is 1.2kb longer than the MRP-8 short arm, that short arm homology is particularly critical in gene targeting experiments.

As reported in the MRP-8 targeting results above, the first MRP-8 transfection produced a correct targeting of 1:384. It was considered possible that exonuclease digestion of the termini might be occurring; this may result in the 800bp of homology in the 5’ region may drop below the 500bp required for homologous recombination, so
reducing the efficiency of correct targeting. Therefore, an attempt was made to limit the impact of this by the use of a hairpin capping oligonucleotide. This was ligated to the linearised knockout construct, with the principle behind this strategy being a loop of non-complementarity at the end of the construct may prevent exonuclease digestion of the 5’ end of the construct. A model for this is shown in Figure 5.3 (on page 196).

This hairpin-protection strategy was followed prior to the second MRP-8 transfection. Unfortunately, it is impossible to say what effect, if any, hairpin ligation had on targeting, which in this case was 1:480. Anything less than a two-fold change in targeting efficiency could not be seen in a sample of 480 neomycin-resistant clones.

There were examples from targeting either MRP-8 or MRP-14 that resulted in correct initial diagnostic profiles but later incorrect profiles; examples include 1C for MRP-14 and 3C11 for MRP-8. As clone 1C was discarded and not used to generate mutant mice, the mechanism and potential impact of the aberrant targeting will not be discussed. However, the MRP-8 clone, 3C11 was used to generate mutant mice.

With reference to Figure 5.9A and Figure 5.9B (on page 207), the EcoRI profile of 3C11 appeared to be normal with a mutant (MUT) band of 2.4kb and a wild-type (WT) band of 2.4kb. This suggested that 5’- targeting was correct. Attempts to investigate the 3’- targeting using probe B were unsuccessful. Initial characterisation experiments, prior to ES transfection suggested that the probe would be functional. Unfortunately, later investigations did not support this. Therefore, the 3’- targeting was investigated, from the 5’- end of the construct with a ScaI digest and probe A (see Figure 5.8, on page 204). This investigation revealed that instead of the expected 9kb (WT) and 10.5kb (MUT) profiling, only a 9kb band was produced (Figure 5.9C). However, when the neomycin probe was used, 2 bands, the expected 10.5kb and a 9kb band (exactly overlaid the probe A 9kb band) were produced (see Figure 5.9D). This suggested two possibilities; firstly, that a replacement event had occurred but that homologous recombination was not perfect, resulting in a loss of 1.5kb sequence (this could account for the 9kb ScaI product); secondly, this suggested that there was a second construct integration in the genome. An insertion at the 5’- end of the gene seemed unlikely; in such a case, it might be expected that the ScaI digest would produce a 14.8kb band (5.5kb of construct plus 9.5kb of genomic sequence).

Given the low targeting frequency of MRP-8, the data suggesting that the first clone, 5FD10 was correctly targeted, the suggestive data that 3C11 was probably a
Chapter 5: MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

replacement event and the presence of 3C11 chimeric mice (by the time the investigations were complete), all contributed to the decision to use the 3C11 mice as ‘backup’ mice to provide confirmatory data for the 5FD10 mice.

5.5.6 Sequencing

One particularly interesting feature of the analysis of the MRP-14 sequence is the putative overlap between the most likely promoter region (1823 to 1873) and the start of exon 1. There may be several trivial explanations for this; the promoter identification programme may have predicted incorrectly; alternatively, there may have been an error in the initial cDNA cloning and sequencing (140) and the bases in question may not belong to the cDNA. The second explanation seems unlikely, given that the sequenced cDNA clone was identified by antibody selection and subsequent hybridisation analysis. However, this investigator is not aware of examples of genes that have a region of the promoter within the cDNA.

The exonic sequences compare very closely with the reported cDNA sequence (140); the only difference found was a single base change in the 3'-untranslated region, at position 4838, from T to A. This seems not to be a sequencing error, on the part of this investigator, as the data is very repeatable and may represent a mutation or strain polymorphism (129sv used for this study, unknown strain in (140)).

A facile comparison of intron lengths revealed a common finding, which is that the murine introns were shorter than the human counterparts (Table 5.5, page 220). As intron 2 was found to contain a simple repeat, (GAA)n, this may have a contributory factor in the inability to PCR between exons 2 and 3 when attempting to generate a long genomic probe.

A recent report has identified an enhancer region in the human MRP-14 gene, located between exons 2 and 3 (181). This region was found to cause a factorial transcriptional increase of 37 fold when enhancing the activity of the TK promoter in L-132 cells. Four protein factors of unknown identity were shown to bind to the enhancer. This region is present in the murine sequence, in approximately the same position and the only difference found in this study was an insertion of 2 T bases.

Given the position of the 2 most likely promoters and a potent enhancer region for MRP-14, plus the complete restriction map of the gene, it should now be possible to
Chapter 5: MRP-8 and MRP-14 knockout construct generation and ES cell cloning.

Plan, with confidence, a strategy to target the gene in a temporal and tissue specific fashion which avoids potential MRP-14 gene regulatory elements.

5.5.7 Summary

In summary, this chapter has described the cloning and basic characterisation of the murine MRP-8 and MRP-14 genes. It has described the detailed genomic structure of the MRP-14 gene and identified repeat and likely promoter regions, which may prove to be useful in future temporal and tissue specific gene deletion approaches. It has also described the generation and transfection into ES cells of interruption-knockout constructs for both MRP-8 and MRP-14 genes. These transfections were successful and correctly targeted clones for both genes were identified for future development.
6. Generation and basic characterisation of MRP-8 and MRP-14 mice.

6.1 Introduction

In chapter 5, the cloning of the MRP-8 and MRP-14 genes, followed by the generation of knockout constructs, transfection of ES cells and identification of recombinant ES cell clones was described. This chapter aims to describe the transition from MRP-8 and MRP-14 recombinant ES cell clones, to knockout mice via the generation of chimeras and heterozygote mice. It also aims to describe the basic phenotyping of the MRP-8 and MRP-14 knockout mice in terms of assessing RNA and protein levels, relative leukocyte levels and for MRP-8 knockout mice, behaviour in basic models of inflammation.

Throughout this chapter, several abbreviations for genotypes will be used. These are WT, for wild-type, HET for heterozygote mutant, KO for homozygote mutant (knockout) and KOC for knockout construct (the DNA targeting construct used to electroporate the cells).

This chapter is essentially split into two, with the production and characterisation of MRP-8 knockout mice described in section 6.2 (page 239) and of MRP-14 knockout mice described in section 6.3 (page 265). The discussion section 6.4 (page 278), will then draw together the two sets of results.

6.2 MRP-8

6.2.1 Chimera generation and germline transmission

All ES cell and blastocyst manipulation for both MRP-8 and MRP-14 was performed at the Clare Hall ICRF unit under the direction of Ian Rosewell, by Ian Rosewell, Mary Ann Jacobs and Stephen Wilson. All transgenic mice and transgenic pairings were generated and cared for at Clare Hall by Peter Haggar and Tracy Crafton. This section describes in detail the process of generating chimeric mice and transmitting the targeted mutation of interest to the germline.

The two 129-derived targeted ES cell clones identified in Chapter 5, 5FD10 and 3C11, were thawed from frozen stocks and grown up in cell culture. ES cells were originally derived from the inner cell mass of day 3.5dpc blastocysts (71, 174). Once the recombinant ES cells had been passaged once, C57/B6 day 3.5 blastocysts were obtained by super-ovulating C57/B6 females, mating with C57/B6 males and harvesting the blastocysts. Chimeric blastocysts were then generated by injecting
approximately 15 129-derived ES cells (which are male cells) into each C57/BL6 blastocyst (male or female). These chimeric blastocysts were then re-implanted into foster female mice, made pseudo-pregnant by mating with vasectomised sterile males. 17.5 days later, the females re-implanted with the chimeric blastocysts gave rise to chimeric offspring. A summary of the blastocyst injections for 3C11 and 5FD10 can be found in Table 6.1 (page 242).

Chimerism is generated because the injected, 129-derived ES cells and the endogenous C57/BL6 blastocyst cells which develop into the chimeric mouse were derived from 2 different sources and were not genetic clones (77). The whole purpose of using ES cells to inject into the blastocyst is that the ES cells can then develop together with the endogenous cells of the mouse and contribute to any or all of the component tissues. It is particularly important that the 129-derived ES cells contribute to the zygote-producing cells so that the targeted mutation (which will eventually give rise to the knockout mouse) can be passed on to progeny, through the germline.

The offspring produced from the injected blastocysts are generally male (approximately 75%). This is because approximately 50% of the blastocysts that were injected are male and all the injected ES cells are male. The 25% increase in the expected frequency of males is generated because the injected ES cells literally, “take over” the injected blastocyst and convert the sex to male. This conversion implies that the injected ES cells have contributed to all the chimera’s tissues, particularly the gonads. In the conversion situation, the male ES cells produce male gonads although some of the mouse is derived from the original cells which are female.

As shown in Table 6.2 (page 242), only around 40% of the injected blastocysts gave rise to live progeny. Of this live group approximately 50% were chimeric, showing the low efficiency of the process. However, of the subgroup that were live and chimeric, the level of chimerism was approximately 80%.

Chimerism in young mice derived from 129 ES cells and C57/BL6 blastocysts was visible to the naked eye, via coat colour, after approximately 7 days. Cells deriving from the C57/BL6 blastocyst gave black fur whereas cells derived from the 129 ES cells produced agouti coloured fur. A typical example of chimeric mice with varying levels of 129 contribution is shown in Figure 6.1A (page 243).

Once the chimeric mice had attained sexual maturity, their coat colour was assessed. Those mice with the highest contributions were chosen to breed with female
chimeras or C57/BL6 mice, in order to transmit the mutation. The highest contribution chimeras were chosen as these had the best chance of passing on the targeted mutation. Clearly, transmission only occurred when cells derived from the 129 ES cells contributed to zygote-producing cells.

9 pairs of mice were generated in order to transmit the 5FD10 and 3C11 mutations. These were either chimera versus C57/BL6, or chimera versus chimera pairs. These pairings used up all the available chimeric females and the 9 best chimeric males. The chimeric males in these matings ranged from 95% to 100% chimerism, as adjudged by coat colour.

The progeny from these pairings were then assessed for coat colour to minimise both the number of mice that had to be tail-snipped and the number of genotyping reactions that had to be performed. Considering a male chimera versus female C57/BL6 pairing, progeny were of 2 main types. If the male zygote was derived from C57/BL6 cells, then the new individual was black, as the female C57/BL6 zygote could only contribute black coat colour. In contrast, if the male zygote was derived from 129 cells, which contributes agouti coat colour, then a brown individual was born. Therefore, brown progeny were a mark of transmission of 129-derived DNA. That is, some of the brown mice should have contained the targeted mutation, selected for in the ES-cell transfection.

Brown mice were either WT or HET for the targeted mutation. 129-derived zygotes from the chimera, which were produced from targeted 129-cells, were 50% WT and 50% mutant at the targeted allele. Therefore, brown mice could be WT (129 zygote WT gene and C57/BL6 zygote WT gene) or HET (129 zygote MUT gene and C57/BL6 zygote WT gene) and therefore all brown mice were genotyped to identify the HET mice.

Genotyping reactions were initially performed using a PCR-based method (section 2.16.1.3, page 93 and Figure 6.1B, page 243). Positively identified heterozygotes were then submitted for Southern hybridisation genotyping to confirm their identity.

Of the 9 pairs of mice set up to transmit the MRP-8 targeted mutation from the 5FD10 clone, only 1 pair produced brown mice. In that case, 1 brown male mouse was the sum total of the brown mice produced. Fortunately, this mouse (Figure 6.2B, page 244), displayed the expected dual banding pattern of 573bp and 535bp in the PCR.
Chapter 6: Generation and basic characterisation of MRP-8 and MRP-14 mice.

reaction and the dual banding pattern of 2.4kb and 2.8kb in a Southern hybridisation, that was expected of a HET. This mouse was subsequently used to expand the colony, by creating further HETs via C57/BL6 pairings. These newly generated HET mice were then used to create HET versus HET pairings in an attempt to generate KO mice.

Four of the 9 pairs of 3C11 chimera versus C57/BL6 mice transmitted the targeted MRP-8 mutation to the germline. HET progeny, diagnosed as described above for 5FD10 transmission (Figure 6.2A), were utilised immediately to create HET versus HET pairings in an attempt to generate KO mice.

In summary, both targeted MRP-8 ES cell clones, 5FD10 and 3C11 produced high contribution chimeras. These chimeras were then successfully used to transmit the targeted MRP-8 gene and generate HET mice.

<table>
<thead>
<tr>
<th>ES Clone</th>
<th>Passaged</th>
<th>BC obtained</th>
<th>BC injected</th>
<th>BC re-implanted / mouse</th>
<th>Mice re-implanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C11</td>
<td>1</td>
<td>120</td>
<td>53</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>5FD10</td>
<td></td>
<td></td>
<td>58</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 6.1: Summary of the numbers of blastocysts (BC) injected to generate MRP-8 3C11 and 5FD10 chimeric mice.

<table>
<thead>
<tr>
<th>ES Clone</th>
<th>Litters born / total mice born</th>
<th>Total chimeras born</th>
<th>% injected BC born</th>
<th>% born chimeric</th>
<th>Chimeric males / females</th>
<th>Average % chimeric coat colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C11</td>
<td>2 / 11/18</td>
<td>11</td>
<td>34</td>
<td>61</td>
<td>9/2</td>
<td>80</td>
</tr>
<tr>
<td>5FD10</td>
<td>4 / 12/24</td>
<td>12</td>
<td>41</td>
<td>50</td>
<td>9/3</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 6.2: Summary of chimeric status and total mice born after ES cell injected blastocysts were re-implanted into foster mothers and allowed to be born naturally.
A. Examples of high-129 contribution and low-129 contribution, MRP-14 129sv-C57/BL6 chimeric mice with a wild-type C57/BL6 mouse.

B. Schematic diagram showing the approximate locations of primers used for the MRP-8 genotyping reaction within the wild-type (WT) and mutant (MUT) alleles. In the WT allele, the G-Neo primer does not bind and only a 535bp product is generated. In the mutant allele the 8G-GB2 primer binds too far away from the reverse primer to generate a product. The G-Neo and 8G-GF1 primers are now located close together and generate a 573bp product.
Figure 6.2: First identifications of MRP-8 5FD10 and 3C11 heterozygotes and homozygotes. Key: H - heterozygote; W - wild type; K - knockout; KOC - knockout construct. A. Identification of first MRP-8 3C11 heterozygotes by PCR and Southern hybridisation with probe A. B. Identification of first MRP-8 5FD10 heterozygotes by PCR and Southern hybridisation with probe A. C. Identification of first MRP-8 knockout mice by PCR and Southern hybridisation with probe A.
6.2.2 Generation of knockout mice and colony management

This section aims to describe the transition from the presence of HET mice within the colony to the presence of WT, HET and KO mice within the colony. It also aims to explain the method used to maintain the mouse colonies after the generation of knockouts.

Throughout the MRP-8 and MRP-14 knockout mouse projects, there was a general tenet in the breeding programs. Although both 129 and C57/BL6 are inbred mouse lines, there is a degree of heterozygosity that prevents the sort of inbreeding depression manifest by small litter sizes and high pup mortality. However, if these mice are sibling-mated, such problems can appear (P. Hagger, personal communication). Therefore an active decision was taken not to use sibling matings wherever possible.

Once HET MRP-8 5FD10 and 3C11 mice had been generated and an agreement between the genotype diagnosis reached when using either Southern hybridisation or PCR, HET versus HET breeding was started in earnest. If MRP-8 was not necessary for normal development then the expected ratio of progeny should have been 1:2:1 (WT:HET:KO). However, HET intercrosses using either 3C11 or 5FD10 mice, lacked knockout mice when the progeny were genotyped by PCR. Moreover, the ratio of WT:HET:KO was approximately 1:2:0 (data not shown). Litter sizes from such HET versus HET intercrosses varied from 9-11, even in litters 2 and 3, which were generally larger than the first (data not shown). Litter sizes can be up to 14 pups (P. Hagger, personal communication). This suggested that pups might be being lost in utero. Taken together this data suggested the hypothesis that the lack of a functioning MRP-8 gene caused failure of development, at an unknown point. Confirmatory Southern analysis of the PCR genotyping results was not performed at this stage.

With the hypothesis that the lack of MRP-8 was lethal in development, a search was initiated to find KO embryos early in development and then work forward in development until the point was reached when the lack of MRP-8 became lethal. This involved initiating timed matings between HET versus HET pairs, removing embryos at defined times, using them to prepare DNA and then genotyping that DNA by PCR. Following this approach, various litters were analysed but in the majority of cases no knockout embryos were found. In one case, at the earliest time point analysed, a 3C11 litter taken at 8.5 days post coitum (dpc) was genotyped and found to consist of 1:6:1 (WT:HET:KO) embryos. The KO embryo genotype is shown in Figure 6.2C. Here,
Chapter 6: Generation and basic characterisation of MRP-8 and MRP-14 mice.

The PCR reaction produced only a single band of 573bp (compared to a single 535bp band for a WT or dual 535bp/573bp bands for a HET). This suggested that MRP-8 was not required for development up to day 8.5 of development.

At this stage, to positively confirm the hypothesis that MRP-8 was indeed necessary for development and that no KO mice were being born it was decided to re-genotype a number of heterozygote intercross litters using Southern hybridisation rather than the PCR genotyping method. Unexpectedly, this analysis showed that KO mice, from both the 3C11 and 5FD10 clones, did exist and were viable. Some mice that had been genotyped as HET by PCR were shown to be KO animals by Southern analysis. An example set of data from 3C11 HET intercrosses is shown in Figure 6.2C. As can be seen from the PCR reactions, a high proportion of mice were HET (dual banding of 573bp and 535bp). However, when these mice were re-analysed by Southern hybridisation, all 3 genotypes, WT (single 2.8kb band), HET (dual 2.8kb/2.4kb bands) and KO (single 2.4kb bands) were present. This finding was repeated with the 5FD10 clone (data not shown).

These data clearly demonstrated that the MRP-8 PCR genotyping reaction was flawed. Therefore, all subsequent genotyping reactions were performed using the Southern hybridisation protocol, rather than the PCR protocol.

Once homozygote WT and KO animals had been identified, the decision was taken to breed single genotypes, rather than interbreeding heterozygotes. That is, to breed WT versus WT and KO versus KO. In this way, once the first litter had been genotyped by Southern hybridisation, there was no need to genotype further litters. This method also had the advantage of minimising the number of wasted animals that would be generated if HET versus HET progeny were used, in experiments that were designed to compare WT and KO animals. However, this method also eliminated the control within an experiment whereby mice that were compared were litter mates. Therefore, such factors as developmental environment and genetic background that were identical in an experiment using litter mates were lost and extra variation could potentially be introduced into an experiment.

In summary, initial data using only PCR genotyping as a diagnostic tool, did not identify any KO animals from HET versus HET intercrosses. This suggested the hypothesis that MRP-8 was required for development. Examination of mouse embryos at various stages of development also did not reveal any KO embryos, except in one
case where one KO embryo was diagnosed by PCR from a day 8.5dpc litter. This supported the hypothesis that MRP-8 might be required in development. Subsequent analysis of the progeny from various 3C11 and 5FD10 HET intercrosses by Southern hybridisation, revealed that KO animals were viable and living within the mouse colony. This disproved the hypothesis that MRP-8 was necessary for development and demonstrated that the PCR genotyping assay was flawed.

6.2.3 Antibody generation

Three antibodies against MRP-8 were generated during the course of this study, 2 rabbit polyclonal antibodies and 1 rat monoclonal antibody. All the work in immunising and harvesting the polyclonal antibodies was done by Del Watling (ICRF). All the work in generating the rat monoclonal antibody was done by Jane Steel (ICRF).

The general scheme for the generation of rabbit polyclonal antibodies was as follows. Prebleeds were taken at day 0, then rabbits were immunised with 200μg of immunogen in incomplete Freund’s adjuvant. Rabbits were boosted a further 6 times with 100μg immunogen at 2 week intervals, with test bleeds taken after the second and fourth boosts. Finally, rabbits were bled out at 13 weeks and 3 days.

Using this protocol and 100μg His-MRP-8 protein (see section 3.3.2, page 114) as the immunogen, a rabbit polyclonal antibody was generated, named NH-7. This antibody was found to react specifically with His-MRP-8 and not with MRP-14 on a Western blot (data not shown). This antibody failed to react with lysates from bone marrow cells (in which 50% of the cells are mature neutrophils with large amounts of MRP-8) in a Western blot and also failed to immunoprecipitate MRP-8 from bone marrow lysate (data not shown). However, NH-7 did stain cells prepared for intracellular staining by FACS (see section 6.2.4, page 248).

Given the failure of the original antibody to respond to MRP-8 in all but one presented form (intracellular FACS staining) it was thought that the immunogen might not be optimal. The reasons for this hypothesis were that His-MRP-8 is electrophoretically slower than predicted in terms of molecular size on SDS-PAGE and this suggested possible protein mis-folding. Also, His-MRP-8 is very unstable (see section 3.3.2, page 114). This may then have resulted in an antibody that did not recognise native mouse MRP-8. Therefore, the whole MRP-8 protein (MRP-8p) was
synthesised by Nicola O'Reilly (ICRF). MRP-8<sub>p</sub> was then used as an immunogen to generate subsequent antibodies.

The second antibody to be generated was a rat monoclonal antibody. This was generated by immunising a rat in a fashion analogous to that used to immunise the rabbit, but with MRP-8<sub>p</sub>. The spleen was then removed and the splenocytes fused to myeloma cells to form a hybridoma. The supernatants from various hybridoma clones were then tested for immunoreactivity in ELISA assays. Finally, the reactive supernatants were tested on Western blots. These clones were then grown up and used to generate large quantities of monoclonal antibody.

Only 1 clone was found that specifically reacted with MRP-8<sub>p</sub>. The tissue culture supernatant produced from this clone was all used in an important transgenic Western blot (see section 6.2.8, page 259). Unfortunately, this clone lost reactivity upon culturing.

The final anti-MRP-8 antibody to be generated was another rabbit polyclonal antibody, NH-9, again raised against MRP-8<sub>p</sub>. Early test bleeds were found to specifically react with MRP-8 in bone marrow cell lysates on Western blots although cross-reactivity with MRP-14 was also detected (see section 6.2.8).

6.2.4 Gross phenotype
This section aims to describe the gross phenotype of the MRP-8 5FD10 and 3C11 transgenic mice by the examination of various features such as body mass and breeding characteristics. All transgenic mice that were used in an experiment were weighed after sacrifice, so that a database of body masses was generated and any differences that may have been due to the genotype, isolated and identified. Mice were included that had undergone procedures, prior to sacrifice, on the grounds that such procedures were too short in terms of time to cause weight change (24h or less) and also, that all the genotypes were subject to the same procedures. The only exception to this was the air pouch procedure, which lasts for 7 days. Mice were weighed before and after the procedure but no differences were observed (data not shown), therefore the data was included in the database.

Examination of the mass of transgenic MRP-8 mice, post-sacrifice, revealed no differences, between the genotypes, sexes or clones (Figure 6.3A, page 250). That is to say, presence or lack of MRP-8 did not affect the adult mass of mice, as adjudged by
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Overlapping mean average ± SEM, within either sex grouping. Age within adulthood (defined as >42 days) did not seem to affect mass to a large degree (data not shown), so data for all adult ages was grouped together for analysis. Age ranges were 90-300 days for 5FD10 females, 42-390 days for 3C11 females, 100-320 days for 5FD10 males and 150-280 days for 3C11 males.

Breeding records were also analysed to see if the targeted MRP-8 gene was inherited according to the standard rules of Mendelian genetics. The breeding statistics for HET intercrosses of either 5FD10 or 3C11 mice were analysed (those intercrosses that had been confirmed by Southern hybridisation). The results are presented in Table 6.4 below. Neither of the clones produced the expected WT:HET:KO ratio of 1:2:1, as expected from a HET versus HET cross by Mendelian genetics. Indeed, there were far fewer HET and KO mice generated than would have been predicted from such rules. However, both WT and KO mice have been successfully bred as single genotype intercrosses, with no noticeable differences in litter sizes and no parenting problems (such as eating of progeny by the parents) (data not shown). Also, up to the age of 16 months, no differences have been observed in the longevity of MRP-8 3C11 mice (5FD10 mice have not been specifically observed) (data not shown). This suggests that although the HET versus HET progeny ratios are disturbed, MRP-8 plays a redundant role (or no role at all) in development. The reasons for such a perturbation in progeny ratios is not clear.

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<td>6:6:3</td>
<td>9:5:3</td>
<td>15:11:6</td>
<td>1:0.73:0.40</td>
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<td>3C11</td>
<td>24:15:10</td>
<td>22:20:17</td>
<td>46:35:27</td>
<td>1:0.76:0.59</td>
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Table 6.3: Summary HET versus HET breeding statistics for MRP-8 5FD10 and 3C11 mice, broken down by sex.

In summary, no gross phenotype was observed after the removal of MRP-8 from the development and subsequent existence of mice, in either the 5FD10 or 3C11 clones. Neither mass, relative vigour, life-span or breeding patterns seemed to be affected, suggesting that MRP-8 was not necessary for normal development.
Figure 6.3: A. Lack of MRP-8 protein has no effect on the mass of adult mice. Clone and sex of mice that have been grouped together are shown in the vertical axis. Points are shown as mean ± SEM (n = 2-14 for MRP-8 5FD10 3C11 WT, HET and KO mice and n = 3-21 for MRP-8 3C11 WT, HET and KO mice). B. Analysis of MRP-8, MRP-14 and HPRT mRNA levels in MRP-8 transgenic mice by hybridisation analysis of an RT-PCR reaction. MRP-8 3C11 KO mice have 9% of the MRP-8 mRNA and 20% of the MRP-14 mRNA that MRP-8 3C11 WT mice possess.
6.2.5 RT-PCR analysis

The first question to be asked about the MRP-8 transgenic animals after gross phenotyping concerned the level of MRP-8 mRNA within the cells. There are two main methods that can be used to evaluate this, Northern blotting and RT-PCR. Northern blotting is a longer, technically more complicated process than RT-PCR. Therefore, it was decided to use RT-PCR to evaluate the relative levels of MRP-8 mRNA and MRP-14 mRNA in both MRP-8-deficient and MRP-14-deficient animals (see section 6.3.5, page 272).

Initial analysis of product levels derived from MRP-8 5FD10 and 3C11 RT-PCR experiments suggested that KO animals expressed about 20% and HET animals about 75% of WT MRP-8 mRNA levels (data not shown). Also, KO and HET animals seemed to express approximately 75% of the WT MRP-14 mRNA levels (data not shown). These experiments used the cDNA primers that were successfully used to clone the MRP-8 and MRP-14 cDNAs.

To further examine MRP-8 mRNA and MRP-14 mRNA expression, a 3C11 RT-PCR experiment was Southern blotted and hybridised sequentially with MRP-8 cDNA, MRP-14 cDNA and HRPT cDNA probes. The signal from the resultant blots was evaluated using a phosphoimager. As HPRT levels should have been the same within each cell, final results were obtained by correcting each signal by the HPRT signal, so that differences in the amount of template being compared were eliminated. Figure 6.3B (page 250) shows that MRP-8 3C11 KG animals expressed 9% WT levels of MRP-8 mRNA whereas HET animals expressed 24% of the WT MRP-8 mRNA levels. Also, MRP-8 3C11 animals had a decreased MRP-14 mRNA levels; KG animals had 20% and HET animals 30% of WT levels.

These results were different from those that had been gained after observation of the RT-PCR reaction on a ethidium bromide gel. This suggested that some of the products generated in the PCR amplification of the cDNA template were not specific. Although 5FD10 RT-PCR experiments were not blotted and hybridised, it is thought that a similar expression pattern to that found in 3C11 animals exists given that the raw RT-PCR reactions, as analysed by agarose gels, looked identical to those generated by 3C11 mice. Time constraints meant that it was more productive to assume the results were the same for both the MRP-8 clones and pursue the evaluation of absolute protein

251
levels. Indeed, later Western analysis showed that MRP-8 protein expression levels were the same in 5FD10 and 3C11 transgenic mice (section 6.2.8, page 259).

In summary, the 3C11 RT-PCR hybridisation results and the 5FD10 RT-PCR results implied that there was still a basal level of MRP-8 mRNA expression in KO animals. This was unexpected, given that the targeting of both clones was thought to be true replacement events (section 5.2.4, page 205). Whether this mRNA was functional, that is, could generate MRP-8 protein remained to be seen and awaited confirmation from protein expression analysis, FACS staining and Western blotting. If MRP-8 protein were to be produced in quantities proportional to the amount of mRNA present, the animals would be hypomorphic for MRP-8 and would not be "knockouts".
6.2.6 FACS analysis

In the previous section, MRP-8 3C11 KO mice were shown to have 9% of WT levels of MRP-8 mRNA. This situation was thought to be mirrored in the other MRP-8 clone, 5FD10. These data suggested that the targeted mutation in the MRP-8 gene was a hypomorphic mutation. That is, “knockout” mice carrying two targeted MRP-8 genes would have a low level, rather than an absence, of protein expression. The ideal method to test whether the targeting approach used with MRP-8 resulted in a null-expression or hypomorphic phenotype would have been Western blotting. Unfortunately, Western blotting was not possible early on in the MRP-8 study as the available anti-MRP-8 antibody, NH-7 did not react with native protein that had been denatured on a Western blot (data not shown).

Although NH-7 did not recognise denatured MRP-8 protein, it was unclear whether it would react with native MRP-8. The first attempt to evaluate NH-7 staining of non-denatured MRP-8 was via intracellular FACS staining. Bone marrow cells were chosen to investigate the intracellular expression of MRP-8 because of their relative quantity within the mouse (approximately 3.5 x 10^7 cells per femur), the high density of neutrophils and developing neutrophils (40-50% mAb 7/4 positive) and consequently suspected high MRP-8 and MRP-14 expression levels, within the bone marrow cells. Initial investigations revealed that NH-7 did indeed stain permeabilised bone marrow cells, in a very similar manner to the anti-MRP-14 antibodies NH-6 and 2B10 (data not shown). NH-7 also stained the same area of the scatter plot as the anti-neutrophil antibody, 7/4 (data not shown). Taken together this data suggested that NH-7 was able to recognise non-denatured MRP-8, specifically.

Bone marrow cells were obtained from 5FD10 and 3C11 WT, HET and KO MRP-8 mice, permeabilised and stained with anti MRP-8 (NH-7 rabbit polyclonal) and anti MRP-14 (2B10 rat monoclonal) antibodies. These cells were then analysed by flow cytometry. As can be seen from Figure 6.4 (page 256), 5FD10 WT bone marrow cells (green trace) could be divided into a MRP-8 expressing and a non-MRP-8 expressing population. Although not represented on the plots, the secondary alone control plot overlaid the non-expressing peak (data not shown). This situation was mirrored for the 5FD10 HET (blue trace), except that the positive peak appeared to be left-shifted, suggesting a decrease in the amount of fluorescence and hence MRP-8 protein, present in those cells. In the KO cells (red trace), there did not appear to be a large positive.
peak of MRP-8 expression, but rather a tail, from the relatively large negative peak. This data suggested that 5FD10 KO cells expressed virtually no MRP-8, HET cells expressed well but less than WT levels.

This situation was broadly mirrored in the 3C11 bone marrow cells, except that the HET positive peak seemed to overlay the WT positive peak, rather than undergo a left shift as was seen for 5FD10, suggesting that HET animals expressed equivalent amounts of MRP-8 as the WT cells.

Examination of MRP-14 expression levels with the rat monoclonal 2B10 antibody suggested that MRP-8 HET and KO animals expressed as much MRP-14 intracellularly as MRP-8 WT animals. This situation was mirrored in both clones, 5FD10 and 3C11.

Further FACS analyses followed, which included both blood and bone marrow cells of both clones. These investigations were very variable and the patterns seen in one investigation were not repeated. As the absolute values obtained from the FACS depend on the instrument, its settings as well as inherent biological variation, the changes seen in each experiment were expressed as a percentage of WT levels. These percentages were then averaged across the experiments.

This form of analysis revealed that there were no changes in any of the parameters measured, as adjudged by overlapping mean averages ± SEM (data not shown). Measured unchanged parameters included, for blood granulocytes: FSC (size), SSC (granularity), % T cells, % B cells, % neutrophils, % Mac-1, % LFA-1, % L-selectin and % Gr-1. For bone marrow cells these parameters were: FSC (size) and SSC (granularity), % monocytes / macrophages, % neutrophils, % Mac-1, % LFA-1 and % L-selectin. Other antibody stainings were performed but not in sufficient numbers to be averaged and so will not be discussed.

In summary, FACS analysis of MRP-8 WT, HET and KO bone marrow cells has shown that MRP-8 5FD10 and 3C11 KO cells express very little or no, MRP-8, as compared to WT bone marrow cells, which were found to express significant amounts of this protein. MRP-8 HET bone marrow cells were found to express approximately WT levels of MRP-8 protein. In contrast to the changes seen in MRP-8 protein levels, no changes were detected in MRP-14 protein expression levels in MRP-8 WT, HET or KO bone marrow cells. In addition, no significant differences could be observed in the
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Cellular size, granularity or composition of either blood or bone marrow cells in the MRP-8 WT, HET and KO 5FD10 and 3C11 mice.
Figure 6.4: MRP-8 5FD10 and 3C11 KO mice do not express MRP-8 but do express MRP-14 as adjudged by intracellular FACS staining. Each plot is referenced in a co-ordinate fashion. Plots are overlaid FACS histogram plots with the key: green - WT, blue - HET and red - KO. In the illustrated experiment, there are 1 WT, 1 HET and 2 KO 5FD10 and 2 WT, 1 HET and 2 KO 3C11 mice represented on the plots.
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6.2.7 Immunoaffinity purification

In the previous section, FACS analysis was shown to suggest that the levels of MRP-8 in 3C11 and 5FD10 KO mice were either almost negligible or null. However, this answer was not definitive and further proofs were sought as to the presence or absence of MRP-8 in the “knockout” mice.

In humans, MRP-8 and MRP-14 are generally found as a heterocomplex (22, 67, 200, 255) although this is less clear in the mouse. However, as discussed in the Introduction, some reports suggest that MRP-8 and MRP-14 can form complexes in vivo (129). Therefore, it was thought that one way to assess the levels of MRP-8 in supposed MRP-8 knockout animals could be indirect; that is, via MRP-14. If MRP-14 forms a complex with MRP-8, then coupling an anti-MRP-14 antibody to a column would bind the putative complex of MRP-14/8 which would then be visible in the eluate. This hypothesis was tested by coupling the rat monoclonal anti-MRP-14 antibody, 2B10 to Sepharose beads and generating an antibody affinity column. To this column was added a lysate derived from MRP-8 WT or MRP-8 KO bone marrow cells.

As can be seen from Figure 6.5A (page 261), the lysate derived from WT 3C11 mice, after it was specifically bound and then eluted from the column, contained 4 bands of 34kDa, 24kDa, 14kDa and 10kDa. The identity of the 10kDa and 14kDa bands were known. Firstly, there are 2 main bands in 4h-thioglycollate elicited cells at 10kDa and 14kDa which MALDI analysis has shown to be MRP-8 and MRP-14 (data not shown). Secondly, 2B10 antibody is a specific MRP-14 reagent, which reacts with a 14kDa band on Western blots (see right hand panel, Figure 6.10C, page 277). Therefore the 14kDa band must have been MRP-14. The most likely candidate to complex with MRP-14, given the human data and the size of the candidate band, was MRP-8. Therefore, from these suppositions, it was hypothesised that the bands may represent MRP-8/14/8 (34kDa), MRP-8/14 (24kDa), MRP-14 (14kDa) and MRP-8 (10kDa), respectively.

Also in Figure 6.5A, it can be seen that the lysate derived from KO 3C11 mice contained only one band, at 14kDa; this was MRP-14. This data supported the intracellular FACS staining data, suggesting that KO 3C11 mice have negligible or zero amounts of MRP-8 protein, at least in association with MRP-14. This data also suggested that the supposed identity of the 10kDa band as MRP-8 and the higher molecular weight species, found in the MRP-8 WT purification were as suggested
above, because the absence of MRP-8 in the MRP-8 KO lysate removed these bands from the eluate.

It was thought that the difference found between the eluates produced from the WT and KO lysates results were not due to any column degradation as the KO lysate binding was done first, followed by the WT lysate. Another possible explanation for the results was that MRP-8 5FD10 and 3C11 mice are indeed hypomorphic and that the low amounts of MRP-8 in these animals do not form complexes with MRP-14 but stay as free protein. As the column does not bind MRP-8 directly, this is not detected in the eluate and is not seen in the flow-through from the column, because it is of too low a concentration to be seen, even by silver stain. Interestingly, no MRP-8 or MRP-14 was seen in the flow through from the WT lysate (data not shown), suggesting that in the WT cells, the vast majority of MRP-8 was complexed to MRP-14, as is the case in humans. Clearly, this data does not preclude MRP-8 WT or KO 5FD10 and 3C11 animals possessing some MRP-8 that does not complex with MRP-14 and is of such a low concentration that it does not appear in the flow through.

These experiments were not repeated with 5FD10 mice because, by this time, a specific Western blot assay had been developed. This was considered the best way to assay absolute levels of MRP-8 protein.

The only problems encountered with the use of this system were that not enough protein to positively identify by MALDI analysis was eluted using a 2ml immunoaffinity column. This was probably not due to column overloading as there was little or no flow through (data not shown) in the purifications. This meant that the identity of the 10kDa band, believed to be MRP-8, and supported by several lines of evidence, could not be directly confirmed. The second problem with the assay system was column instability. If a column was left for more than a week between bead coupling and use, or, if an attempt was made to re-use a column that had been used once before (as in, 2 purifications from an experiment as described above had been performed) then no eluate could be obtained. This suggested that the harsh 1mM HCl wash may have denatured the antibody or that the rat monoclonal antibody was itself inherently unstable. Therefore, columns were generated as they were needed.

In summary, when the bone marrow cell lysate from MRP-8 3C11 WT mice was purified using an anti-MRP-14 monoclonal antibody, several bands, thought to be complexes of MRP-14 and MRP-8 were eluted. In contrast, when the lysate from
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MRP-8 3C11 KO mice was purified using the same column, no MRP-8 complexes but only a single MRP-14 band was eluted. No MRP-8 or MRP-14 was detected in the flow through from either purification. This data suggests that the majority of MRP-14 and MRP-8 do form complexes in vivo and also that MRP-8 3C11 KO mice have very low or absent levels of MRP-8, as compared to MRP-8 3C11 WT mice.

6.2.8 Western blot analysis of MRP-8 and MRP-14 expression levels in MRP-8 WT and KO mice.

It is thought that the best way to assess whether a protein is present or not, in a putative knockout, is via the use of a Western blot. Early on in the MRP-8 study, it was not possible to do this because NH-7, the only antibody available, did not react with denatured MRP-8 protein on a Western blot although it seemed to react with non-denatured MRP-8 protein by FACS (see section 6.2.4, page 248). Various attempts were made to mimic the reactivity of NH-7 in FACS with denatured, blot-associated MRP-8 including optimising protein transfer conditions, changing blocking agents and altering incubation times but these attempts were not successful (data not shown).

Eventually, after the synthesis of the MRP-8 as a peptide, and its use as an immunogen to create a rabbit polyclonal antibody, NH-9, a breakthrough was made. A test bleed of this antibody was shown to react relatively specifically with denatured native MRP-8 (1:100 test bleed I), present on blots as part of a general bone marrow lysate. The other, less-reactive species was MRP-14 (data not shown). Therefore, the absolute MRP-8 levels in both knockout clones could finally be definitively measured.

Two WT and three KO mice from each clone, 5FD10 and 3C11 were sacrificed and bone marrow lysates prepared (as described in section 2.20.3, page 103, except that lysates were prepared for each mouse and lysate was made from 5x 10^6 cells in a total volume of 100μl). 30μl (1.5 x 10^6 cells) of lysate was then run in each lane of a Western blot and 15μl (0.75 x 10^6 cells) of lysate per lane used for a normal gel. As can be seen from Figure 6.5B (page 261), a silver stained 15% SDS-PAGE gel, MRP-8 was apparent in the WT but not the KO lysates, derived from either 5FD10 or 3C11 animals.

When a Western analysis was performed, probing with a test bleed of the anti MRP-8 antibody, NH-9, as shown in Figure 6.5C, the identity of the approximately 10kDa band was confirmed as MRP-8. Both WT mice from 3C11 and 5FD10 (second WT has a band that is faint but present) had MRP-8 protein but none of the knockout
mice from either 3C11 or 5FD10 clones contained any MRP-8. The exposure shown is an early one (2 minutes), in order to minimise the background. Later exposures, of up to 10 minutes failed to reveal any 10kDa bands in the KO lanes. This data suggested that both MRP-8 clones were true knockouts as the tissue in the body where most MRP-8 could be found was analysed and found to lack MRP-8 expression.

Further analyses using rabbit antibody NH9 confirmed that MRP-8 KO mice did not express any MRP-8 in bone marrow lysates and also showed that MRP-14 protein levels were decreased by approximately 50%, as compared to WT mice (Figure 6.10C, page 277). This suggested that MRP-8 might regulate the expression of MRP-14 in a positive manner.

It is important to note that the expression profiles of MRP-8 and MRP-14 were the same in both the 5FD10 and 3C11 clones. After the identification of targeted MRP-8 ES cell clones, concerns were raised over the correct targeting of the MRP-8 gene in the 3C11 clone (see section 5.2.4, page 205). Given that the above protein expression results are the same for both the 5FD10 clone and the 3C11 clone, this does not appear to be significant.

In summary, this section has shown that MRP-8 5FD10 and 3C11 KO mice, in contrast to MRP-8 5FD10 and 3C11 WT mice, did not express any MRP-8 protein in their bone marrow. Moreover, MRP-8 protein expression seemed to be permissive for MRP-14 protein expression as, in MRP-8 KO bone marrow, MRP-14 protein levels were decreased to approximately 50% of the MRP-14 protein levels found in MRP-8 WT bone marrow cells.
A. Anti-MRP-14 monoclonal antibody immunoaffinity purification using bone marrow cell lysates from 3C11 WT and KO mice.

B. Bone marrow cell lysates from 5FD10 and 3C11 KO and WT mice were run on a 15% SDS-PAGE gel and silver stained.

C. Western blot of bone marrow cell lysates derived from MRP-8 5FD10 and 3C11 WT and KO mice, probed with a new anti MRP-8 polyclonal antibody test bleed.

Figure 6.5: MRP-8 3C11 and 5FD10 KO mice have undetectable levels of MRP-8 protein. A. Anti-MRP-14 monoclonal antibody immunoaffinity purification using bone marrow cell lysates from 3C11 WT and KO mice. B. Bone marrow cell lysates from 5FD10 and 3C11 KO and WT mice were run on a 15% SDS-PAGE gel and silver stained. C. Western blot of bone marrow cell lysates derived from MRP-8 5FD10 and 3C11 WT and KO mice, probed with a new anti MRP-8 polyclonal antibody test bleed.
6.2.9 Phenotype of MRP-8 KO mice in inflammatory models

Once preliminary phenotypic characterisation of MRP-8 5FD10 and 3C11 mice had been carried out, such as RT-PCR, FACS, immunoaffinity and Western blotting analyses, it was decided to attempt to answer another of the original aims of the thesis. What is the role of MRP-8 in the inflammatory process? To ask this question, two models were examined, the air pouch model (as used to characterise the in vivo function of MRP-14) and the thioglycollate-induced peritonitis model (as used to generate leukocytes for use in the in vitro profiling of MRP-14 function). The data generated from these models and described below is preliminary and awaits confirmation from future investigations.

Results from an air pouch investigation, using groups of 3 3C11 MRP-8 mice (of each genotype) are shown in Figure 6.6A (page 264). Background inflammation, defined as the response to PBS-cations-Triton X-114, was very similar across the genotypes. The response to TNF was also very similar across the genotypes, at approximately 6 times background levels. In contrast, the response to 1μg LPS was as expected in the WT group (10 times background inflammation) but was much lower in the HET and KO groups (approximately 4 times background inflammation). This may suggest that MRP-8, in myeloid cells, is important in the transduction of the LPS inflammatory response. A recent report has identified an association between LPS signal transduction and S100A proteins. 12 hours after infusion of LPS, a 3.6-fold increase in EN-RAGE (MRP-6) was detected (102).

The response to MRP-14 was dramatically different between the 3 genotypes. WT and HET mice behaved almost as expected (although the HET response was low) with approximately 3 times background inflammation levels. The response of the KO mice to MRP-14 was dramatic and strong, with infiltrate levels at 17 times background levels. This massive response may indicate the importance of the murine MRP-8/14 heterocomplex, that is so well characterised in humans. If MRP-14, demonstrated as an in vivo chemoattractant (see section 4.4.1, page 166) is deactivated by complexing with endogenous MRP-8, released from infiltrating neutrophils, then it is easy to imagine the following scenario. As MRP-8 deficient neutrophils have no MRP-8, they cannot complex the MRP-14 present within the air pouch. Therefore, the air pouch MRP-14 constantly recruits cells, generating the large response seen in the KO but not the WT mice.
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Analysis of the cell populations recruited to various stimuli in the air pouch showed no differences based on the genotype of the animals being investigated (data not shown). In general, as was found in the MRP-14 investigations using the air pouch model TNF, MRP-14 and LPS recruited 70% neutrophils and 30% monocytes / macrophages (data not shown). The cellular make-up of these populations did not vary with the size of the infiltrate generated (data not shown).

The second inflammatory model to be investigated was the thioglycollate-elicited peritonitis model. These investigations mainly used 5FD10 mice but 3C11 mice were also used. Experiments were generally performed with groups of 3 animals; data is presented as a summary of these investigations in Figure 6.6B (page 264).

The data suggests that MRP-8 deficient mice are limited in their ability to recruit leukocytes at early timepoints, 2 (preliminary data) and 4 hours. The 4 hour data is more definite, given that it is the summary of 3 experiments (n=10), and that the mean average ± SEM do not overlap (WT infiltrate $3.70 \times 10^6 \pm 0.52 \times 10^6$, KO infiltrate $2.23 \times 10^6 \pm 0.48 \times 10^6$). However, by 24 hours, the difference in recruitment between WT and KO mice had disappeared, suggesting that the presence of MRP-8 is important only for the early infiltration of myeloid cells (n=6, experiments=2).

Infiltrating cell types were not analysed at the 2 hour timepoint. However, at the 4 hour timepoint there was no detectable difference between the WT and KO animals. Both WT and KO animals recruited approximately 70% neutrophils, 15% monocyte / macrophages and 15% B-cells (data not shown). At the 24 hour timepoint, where no genotype versus infiltrate size differences were observed both WT and KO animals recruited 60% neutrophils, 30% monocytes and 10% B-cells (data not shown).

In summary, this section has demonstrated that differences in response size and reaction times to inflammatory stimuli, do exist between MRP-8 WT and KO animals in both the air pouch and thioglycollate-induced peritonitis models. These are most marked for the MRP-14 induced inflammation in the air pouch, where MRP-8 KO animals recruit many more cells than MRP-8 WT animals and also at 4h in the thioglycollate-induced peritonitis model, where MRP-8 KO animals recruit fewer cells than the MRP-8 WT animals.
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A.

Figure 6.6:- A. Lack of MRP-8 causes a decreased inflammatory response to LPS, an increased inflammatory response to MRP-14 and an unchanged inflammatory response to TNF, in the air pouch model of inflammation. Points are shown as mean ± SEM (n=3 MRP-8 3C11 mice per point). B. Lack of MRP-8 causes a decreased inflammatory response in the thioglycollate-elicited peritonitis model at 4 hours but does not affect earlier or later timepoints. Points are shown as summary means ± SEM (n=2-10 MRP-8 3C11 or 5FD10 mice per point, from 1 (2h timepoint), 2 (24h timepoint) and 3 experiments (4 hour timepoint)).
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6.3 MRP-14

6.3.1 Chimera generation and germline transmission

MRP-14 chimeras were generated in an identical manner to that described for the MRP-8 chimeras (section 6.2.1, page 239). The first clones to be injected were identified from the first transfection of ES cells with the MRP-14 neomycin KOC, namely 12H and 8C. Summary injection statistics for all MRP-14 clones used can be found in Table 6.4 (page 266).

Both the 12H and 8C clones generated viable chimeras, although the coat contribution from clone 8C was inferior to that from 12H clone, 75% versus 82%, respectively. Summary chimera statistics for all MRP-14 chimeras generated can be found in Table 6.5 (page 266). As was performed for MRP-8, 9 pairs of mice were set-up for each clone (chimera versus C57/BL6). However, none of the 8C chimeric mice transmitted brown coats to their offspring, suggesting that the 129-derived ES cells and hence the targeted MRP-14 gene, had not contributed to the zygote-forming tissues of the chimeras. In the case of the 12H clone, only 1 pair transmitted brown colouring through the germline, to the progeny. Analysis of this clone’s transmission will be undertaken in the next section 6.3.2 (page 267).

Two other clones, 3B and 12G, from the first MRP-14 ES cell transfection, were injected into blastocysts. Clone 3B was injected twice, 3B(1) and 3B(2). Unfortunately, neither injection produced a chimera (>60%) adjudged good enough to attempt germline transmission. Clone 12G was more successful, in that breeding-standard chimeras were produced. One chimera versus C57/BL6 pair did transmit coat colour through the germline to produce brown progeny. The analysis of the transmission of this clone is also discussed in section 6.3.2.

A second ES cell transfection with the MRP-14 neomycin construct was performed. This transfection (section 5.3.4, page 213) was even more successful than the first, yielding 13 correctly targeted clones from 196 assayed clones. From this group of 13, 2 clones, 2E5 and 2G1, were injected into day 3.5dpc blastocysts. These injected blastocysts gave rise to chimeras with an average coat contributions of 70% and 64%, respectively. These mice were than paired to C57/BL6 mice in the usual fashion.

Four of the 9 2E5 chimera versus C57/BL6 pairs transmitted coat colour to the germline and all four lines generated HET mice, as assayed by Southern hybridisation and PCR genotyping (for method explanation see section 2.16.2.3, page 94 and Figure
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In contrast, only 1 of the 2G1 chimera versus C57/BL6 pairings transmitted through the germline to produce brown progeny although this was enough to generate HET mice. Southern hybridisation data for the germline transmission of both the 2E5 and 2G1 clones is shown in Figure 6.8A (page 271). In Southern hybridisations, HET mice produced a double band of 4kb and 9kb whereas WT mice produce a single band of 4kb.

HET mice from both the 2G1 and 2E5 clones were subsequently used to expand the colony via the setting-up of HET versus HET intercrosses.

<table>
<thead>
<tr>
<th>ES Clone</th>
<th>Passage number</th>
<th>BC obtained</th>
<th>BC injected</th>
<th>BC re-implanted / mouse</th>
<th>Mice re-implanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>12H</td>
<td>4</td>
<td>67</td>
<td>67</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>8C</td>
<td>4</td>
<td>70</td>
<td>70</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>3B (1)</td>
<td>?</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>12G</td>
<td>?</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>3B (2)</td>
<td>4</td>
<td>37</td>
<td>37</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>2E5</td>
<td>1</td>
<td>36</td>
<td>36</td>
<td>12</td>
<td>3</td>
</tr>
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<td>2G1</td>
<td>3</td>
<td>39</td>
<td>39</td>
<td>13</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 6.4:- Summary of the numbers of blastocysts injected to generate the various clones of MRP-14 chimeric mice.

<table>
<thead>
<tr>
<th>ES Clone</th>
<th>Litters born</th>
<th>Total chimeras born / total mice born</th>
<th>% injected</th>
<th>% born chimeric</th>
<th>Chimeric males / females</th>
<th>Ave. % chimeric coat colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>12H</td>
<td>4</td>
<td>19 / 29</td>
<td>44</td>
<td>66</td>
<td>17 / 2</td>
<td>82</td>
</tr>
<tr>
<td>8C</td>
<td>3</td>
<td>7 / 17</td>
<td>22</td>
<td>41</td>
<td>7 / 0</td>
<td>75</td>
</tr>
<tr>
<td>3B (1)</td>
<td>1</td>
<td>4 / 6</td>
<td>40</td>
<td>67</td>
<td>?</td>
<td>15</td>
</tr>
<tr>
<td>12G</td>
<td>1</td>
<td>4 / 7</td>
<td>46</td>
<td>70</td>
<td>4 / 0</td>
<td>70</td>
</tr>
<tr>
<td>3B (2)</td>
<td>1</td>
<td>1 / 2</td>
<td>17</td>
<td>50</td>
<td>1 / 0</td>
<td>50</td>
</tr>
<tr>
<td>2E5</td>
<td>3</td>
<td>13 / 18</td>
<td>50</td>
<td>72</td>
<td>11 / 2</td>
<td>70</td>
</tr>
<tr>
<td>2G1</td>
<td>2</td>
<td>8 / 16</td>
<td>41</td>
<td>50</td>
<td>8 / 0</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 6.5:- Summary of chimeric status and total mice born after ES cell injected blastocysts were re-implanted into foster mothers and allowed to be born naturally.
6.3.2 Generation of knockout mice and colony management

This section aims to describe the transition from the presence of HET mice to the presence of WT, HET and KO mice within the mouse colony.

As has been discussed in the preceding section, 7 MRP-14 clones were injected into blastocysts to generate chimeric mice although only 2 lines, 2E5 and 2G1, successfully transmitted to the germline and generated heterozygotes. Two of the 5 MRP-14 chimeras that did transmit brown coat colour but did not transmit the mutant MRP-14 gene will be discussed in some detail below. The other 3 did not transmit at all and so will not be discussed further.

The first clone to be injected, 12H, produced chimeras with a good coat colour (average 82%). Of the 9 chimera versus C57/BL6 pairs set-up, only 1 pair produced brown mice (although this was 100% brown progeny with no black littermates); according to Mendelian genetics, 50% of these mice should be HET. Given that all offspring were brown, this suggested that all the zygotes produced from this male were derived from the 129 ES cells and not the C57/BL6 blastocyst. The genotyping of these mice was done by PCR (this was before the MRP-8 PCR was found to be defective, so casting doubt over PCR genotyping, generally). Of 26 brown mice from a total of 5 litters, produced from the 12H transmitting chimera, all were diagnosed as WT (see Figure 6.7B on page 270).

Given that at least 13 of these mice were supposed to be HET, this suggested that MRP-14 might be so important for development that the lack of 1 functional allele may lead to embryonic lethality, as has been found for 1 other gene so far, vascular endothelial growth factor (VEGF) (41). This was tested by the embryo genotyping of progeny from timed matings. No HET embryos were found as far back as 8.5dpc.

Once the transmitting 12H chimeric male had stopped fertilising females successfully, it was sacrificed and DNA prepared from the testis and chordal epididymis. These tissues were then analysed by Southern hybridisation for the presence of the MRP-14 mutant gene. As can be seen from Figure 6.7B, the mutant gene was not present in the gonads. Given that this mouse only produced brown progeny, which suggested that the gonads were fully 129-derived, it might have been expected that the tissue would resemble a HET in its Southern hybridisation profile. This was not the case, the tissue resembled a WT, which neatly explains the 100% brown mice with no HET progeny results. This finding implies that either 12H was a
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mixed clone (a correctly targeted clone with some WT ES cells mixed in) or that 12H spontaneously lost the mutant MRP-14 gene during culture and prior to injection.

The fourth clone from the first transfection to be injected was 12G. This clone produced poor chimeras (average coat contribution 64%) although the 5 best males were used to generate 5 chimera versus C57/BL6 pairs in an effort to transmit the gene. 1 of the 5 pairs did generate brown mice, some of which appeared to be HET by PCR. This is demonstrated in Figure 6.7C (page 270) where in contrast to 129 (WT) DNA which produced a 300bp band or KOC DNA (mimics the KO situation) which produced a 100bp band, several 12G mice produced the banding expected of a HET, which is dual banding of 300bp and 100bp. However, when this result was re-examined using Southern hybridisation, all the 12G mice thought to be HET by PCR analysis were subsequently found to be WT. No progeny were found that were HET by Southern hybridisation and so the breeding program was terminated. This suggested that 12G had lost the correctly targeted MRP-14 gene, identified by Southern hybridisation screening. It also suggested that either 2G1 possessed a second, non-targeted MRP-14 KOC integration in the genome, which created the false positives, picked up by the PCR reaction or that the PCR genotyping reaction was flawed.

After the second transfection, two chimeric lines were established, 2E5 and 2G1. Both lines transmitted through the germline to generate HET mice, as was discussed in the previous section. HET intercrosses were immediately set-up for both clones. 2E5 generated all 3 genotypes, as expected from a non-lethal gene HET intercross; an example Southern analysis of this event is shown in Figure 6.8B (page 271). As can be seen, KO mice have a single 9kb band whereas HET mice have a dual 4kb 9kb banding and WT mice have a single 4kb banding. These results were subsequently verified in a randomised fashion using the PCR genotyping system (Figure 6.8B), verifying that the PCR system was indeed fully functional. KO mice were visualised as single 100bp bands, HET as dual 100bp / 300bp bands and WT as single 300bp bands.

The second clone, 2G1, has subsequently produced KO mice, in a very similar fashion to that seen for 2E5 (data not shown). However, since the PCR assay has been effectively validated using the 2E5 mice and clone targeting and transmission were validated by Southern hybridisation, the decision was taken to perform this and all future genotyping, for both 2G1 and 2E5 by PCR, rather than Southern hybridisation.
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In summary, despite initial transmission problems, the introduced mutation in MRP-14 transmitted through the chimeras to the germline with two clones, 2E5 and 2G1, derived from the initial transfection. The generated HET mice from clones 2E5 and 2G1 then went on to produce KO mice in the fashion predicted by Mendelian genetics.

6.3.3 Antibody generation

Two anti-MRP-14 antibodies were generated in the course of this study, 1 rabbit polyclonal and 1 rat monoclonal. The general methods by which the rabbit and rat antibodies were produced have been described, in section 6.2.3 (page 247). Both antibodies were raised against recombinant MRP-14, produced as described in section 3.4.2 (page 119).

The first antibody to be generated was a rabbit polyclonal antibody called NH-6. This antibody was found to react specifically, by Western blot analysis, with recombinant MRP-14, His-MRP-14 and MRP-14, as present in bone marrow cell lysates (data not shown). It was also found to react by FACS with permeabilised bone marrow cells, and was used in early efforts to characterise the expression levels of MRP-8 and MRP-14 in the MRP-8 KO mice (see section 6.2.4, page 248).

The second antibody to be generated was a rat monoclonal antibody called 2B10. This antibody was found to have the same specificity as NH-6, but was used in preference to NH-6 as it produced “cleaner” data (data not shown).
Figure 6.7: A. Schematic diagram showing the approximate locations of primers used for the MRP-14 genotyping reaction within the wild-type (WT) and mutant (MUT) alleles. In the WT allele, the G-Neo does not bind and only a 380bp product is generated. In the mutant allele the 14G-GF1 primer binds too far away from the reverse primer to generate a product. The G-Neo and 14G-GB1 primers are now located close together and generate a 100bp product. B. Failure to identify transmission of the 12H mutation by tail PCR and Southern hybridisation of transmitting chimera with probe E. C. Putative transmission of the 12G mutation by tail PCR but failure to confirm this diagnosis by Southern hybridisation with probe E.
Figure 6.8: First identifications of MRP-14 2G1 and 2E5 heterozygote and homozygotes. Key: H - heterozygote; W - wild type; K - knockout; KOC - knockout construct. A. Identification of first MRP-14 2G1 and 2E5 heterozygotes by PCR and Southern hybridisation with probe E. B. Identification of first MRP-8 2E5 homozygote knockouts by PCR and Southern hybridisation with probe A.
6.3.4 Gross phenotype

The gross phenotype of MRP-14 2E5 and 2G1 mice was analysed in an analogous fashion to that described for MRP-8 5FD10 and 3C11 mice (section 6.2.4, page 248). From the limited amount of data available for the MRP-14 2E5 mice (n=2-5), it seems that genotype had no effect on the post-sacrifice mass of mice in either sex, as adjudged by overlapping mean average ± SEM (Figure 6.9A, page 274). This data was similar to that found for the MRP-8 5FD10 and 3C11 mice, in that genotype did not affect adult mass, in either sex (ages ranged from 69-110 days). Whether relative adult age or neonate to adult development is affected by the absence of MRP-14 remains to be seen, as not enough mice have been analysed. MRP-14 2G1 mice await initial investigations.

In contrast to the results found for MRP-8, MRP-14 HET versus HET intercrosses generated almost Mendelian ratios (1:2:1). As can be seen in Table 6.6, below, MRP-14 2E5 HET versus HET intercrosses produced an overall ratio of 1:1.56:1.08. The effect of the absence of MRP-14 on longevity, parenting or breeding remains to be investigated.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2G1</td>
<td>0:10:3</td>
<td>1:6:4</td>
<td>1:16:7</td>
<td>1:16:7</td>
</tr>
</tbody>
</table>

Table 6.6: Summary HET versus HET breeding statistics for MRP-14 2E5 and 2G1 mice, broken down by sex.

In summary, initial investigations of MRP-14 2E5 mice have shown that absence or otherwise of MRP-14 has no effect on mass, post-sacrifice. Moreover, MRP-14 mice breed in a fashion predicted by Mendelian genetics, suggesting that no losses are occurring in utero, and suggesting that MRP-14 is not necessary, or is redundant, during development.

6.3.5 RT-PCR analysis

RT-PCR analysis was performed on MRP-14 2E5 animals, in essentially the same way as has been described for the MRP-8 5FD10 and 3C11 animals, except that Southern hybridisation of MRP-14 2E5 with various cDNA probes, was not performed. This
was due to time constraints and the fact that Western blots had already confirmed that protein was indeed absent in KO but not HET or WT animals (see section 6.3.6, page 275). Clearly, it is something that does need to be completed in the future.

Analysis of MRP-14 2E5 relative mRNA levels was performed using internal and external MRP-8 and MRP-14 exonic primers. Analysis of MRP-14 2G1 animals has yet to be performed, as animals were not available until experiments had finished. External exonic primers were those that had been used to clone the cDNAs (including both start and stop codons) and had been found to generate some non-specific products, whereas the internal primers were those used to clone the genomic probes (that is, in exons 2 and 3 for MRP-8 and exon 3 for MRP-14) and had not been found to generate non-specific products. The two sets of primers were also used to assist in the determination of whether truncated mRNA species were produced.

Results from these RT-PCR analyses are shown in Figure 6.9B (page 274). The equal intensity of the HPRT bands demonstrated that template dosing throughout the genotypes was approximately the same. As might be expected, there was no MRP-14 mRNA in the KO animals, and approximately WT levels in the HET, as adjudged from use of the external MRP-14 primers. However, using the internal primers (situated in exon 3), there did appear to be 10-20% WT levels of that portion of the mRNA in MRP-14 KO animals. This could be a truncated product, generated in small amounts as a non-functional product. It is unlikely to be a splice variant as it has been reported that MRP-14 mRNA exists as a single species (140).

Examination of the products generated from the MRP-8 primers, both internal and external showed that the lack of MRP-14 mRNA did not affect the levels of MRP-8 mRNA and that, in fact, there were no discernible differences between MRP-14 WT, HET and KO animals. This is in contrast to the situation found in the MRP-8 KO where lack of MRP-8 mRNA down regulates the level of MRP-14 mRNA. It may be, after hybridisation analysis that this situation is indeed mirrored. Therefore, these results should be treated as provisional.

In summary, the targeting of the mouse MRP-14 gene results in a complete abrogation of MRP-14 mRNA expression and the expression of a possibly truncated mRNA species. MRP-8 mRNA levels appear to be unaffected. All conclusions remain provisional until hybridisation reactions are performed.
Figure 6.9: A. Lack of MRP-14 protein has no effect on the mass of 2E5 adult mice. Clone and sex of mice that have been grouped together is shown in the vertical axis. Points are shown as mean ± SEM (n = 2-5 for MRP-14 2E5 WT, HET and KO mice). B. Analysis of MRP-8, MRP-14 and HPRT mRNA levels in MRP-14 transgenic mice. RT-PCR analysis of MRP-14 2E5 KO mice. KO mice have virtually no MRP-14 mRNA and approximately WT levels of MRP-8 mRNA.
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6.3.6 Western blot analysis of MRP-8 and MRP-14 protein expression levels in MRP-14 WT and KO mice.

The first protein expression analyses to be performed when MRP-14 2E5 KO animals were obtained were SDS-PAGE visualisation and Western blotting. Bone marrow lysates were prepared as described in section 2.20.3 (page 103), except that lysates were prepared for each mouse and the lysate was made from $5 \times 10^6$ cells in a total volume of 100μl. 30μl (1.5 x $10^6$ cells) of lysate was then run in each lane of a gel destined to be used for a Western blot and 15μl (0.75 x $10^6$ cells) of lysate per lane was used for a normal gel.

As can be seen in Figure 6.10A (page 277), which is a silver stained 15% SDS-PAGE gel, there are 2 highly-expressed bands at approximately 14kDa in the WT. It seemed that the higher of the 2 bands decreased in intensity in the HET and disappeared in the KO. A similar pattern was seen for the approximately 10kDa band previously shown to be MRP-8.

Western analysis of these lysates confirmed the suppositions made from analysing the silver stained gel. As can be seen in Figure 6.10B (left panel), which is a Western blot of bone marrow lysates, probed with a test bleed from an anti-MRP-8 monoclonal rat antibody, MRP-8 was well-expressed in the WT, but was found at approximately 50% levels in the HET and was absent in the KO. Figure 6.10B (right panel), a Western blot of bone marrow lysates probed with the anti-MRP-14 rat monoclonal antibody, 2B10, shows that MRP-14 expression exactly paralleled that of MRP-8. MRP-14 was well expressed in the WT, expressed at approximately 50% WT levels in the HET and was absent in the KO. This suggested that the MRP-14 2E5 KG mice did not express MRP-14, confirming their status as KG mice. Taken together, these data suggested that MRP-8 could not be expressed without MRP-14, as absence of MRP-14 expression caused absence of MRP-8 expression. MRP-14 2G1 WT, HET and KO expression levels have not yet been analysed as thesis writing had started before the mice were available.

Further work was done to compare the expression levels of MRP-8 and MRP-14 between the MRP-8 KO and MRP-14 KO animals. Figure 6.10C (left panel), a Western blot probed with a test bleed of the anti-MRP-8 rabbit polyclonal antibody, shows that MRP-8 levels in the MRP-8 5FD10 WT lysate were approximately equal to the MRP-14 2E5 WT levels and approximately 50% greater than those found in the
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Neither the MRP-8 5FD10 KO lysate nor the MRP-14 2E5 KO lysate contained any MRP-8.

When MRP-14 levels were analysed (Figure 6.10C, right panel), in a Western blot probed with the anti-MRP-14 rat monoclonal antibody, 2B10, it was found that the MRP-8 5FD10 WT lysate and the MRP-14 2E5 WT lysate contained approximately equal, large amounts of MRP-14. In contrast, the MRP-14 2E5 KO lysate contained no MRP-14. However, the MRP-14 2E5 HET lysate was found to contain approximately 50% of MRP-14 2E5 WT levels of MRP-14 and approximately the same amount of MRP-14 as the MRP-8 5FD10 KO.

In summary, MRP-14 2E5 KO mice expressed neither MRP-14 protein nor MRP-8 protein, as adjudged by Western blot analysis. In contrast, MRP-8 5FD10 KO animals expressed no MRP-8 protein but approximately 50% WT levels of MRP-14 protein. This suggested a close relationship between MRP-8 and MRP-14, such that MRP-8 and MRP-14 could each regulate the expression of the partner molecule. However, the data suggested that although MRP-14 is required for MRP-8 expression, MRP-8 is not required for MRP-14 expression and that MRP-8 is merely a partial regulator of MRP-14 expression levels.
Figure 6.10 - MRP-14 2E5 KO mice have undetectable levels of MRP-14 and MRP-8 protein whereas MRP-8 5FD10 KO mice have undetectable levels of MRP-8 and 50% WT levels of MRP-14 protein. A. Bone marrow cell lysates from MRP-14 2E5 WT, HET and KO mice were run on a 15% SDS-PAGE gel and silver stained. B. Western blot of bone marrow cell lysates derived from MRP-14 2E5 WT, HET and KO mice, probed with a new anti-MRP-8 monoclonal antibody and a monoclonal anti-MRP-14 antibody. C. Western blot of bone marrow cell lysates derived from MRP-14 2E5 WT, HET and KO mice and MRP-8 5FD10 WT and KO mice, probed with a new anti MRP-8 polyclonal antibody and a monoclonal anti-MRP-14 antibody.
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6.3.7 FACS analysis

Intracellular staining of MRP-14 WT, HET and KO bone marrow cells was not performed, because good data about the expression levels of MRP-8 and MRP-14 had already been obtained from Western blot investigations. Also, Western data is considered more reliable than FACS data, as a measure of absolute protein levels and as a measure of absolute presence or absence of protein.

Preliminary investigations were initiated into the relative cellular composition of the blood and bone marrow compartments of MRP-14 2E5 WT, HET and KO mice, in an analogous manner to those undertaken to determine the cellular composition of MRP-8 WT, HET and KO animals (see section 6.2.6, page 253). As was found with the MRP-8 WT and KO FACS investigations, a large variability, but no definite changes in relative cellularity were observed. No investigations have been initiated with the MRP-14 2G1 mice. Both the MRP-14 2E5 and 2G1 investigations are incomplete and need to be expanded before definitive conclusions about any differences in relative cellularity can be made.

6.4 Discussion

6.4.1 General conclusions

This chapter has described the generation and basic characterisation of MRP-8 and MRP-14 deficient (knockout) mice. Two separate clones of MRP-8 knockout mice were generated; these mice were viable, healthy and bred normally. No differences in gross phenotype were observed. MRP-8 KO mice were shown to express 9% of WT levels of MRP-8 mRNA and 20% of WT levels of MRP-14 mRNA, by hybridisation of RT-PCR reactions. This decrease in mRNA equated to an absence of MRP-8 protein and a 50% decrease in WT levels of MRP-14 protein, as measured by Western blotting. No significant differences in the cell populations of the blood or bone marrow compartments were observed between WT or KO MRP-8 mice. Preliminary investigations into the role of MRP-8 in inflammation, suggested that MRP-8 may be involved in the early entry of leukocytes into the peritoneum as MRP-8 KO mice were slower to react to peritoneal insult than WT mice.

Two separate clones of MRP-14 knockout mice, 2E5 and 2G1, were also generated. Preliminary data showed that these mice were healthy and viable and that no
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Differences in gross phenotype could be detected. Only 1 clone, 2E5, has been analysed. This clone was found to express no MRP-14 mRNA but WT levels of MRP-8 mRNA, by RT-PCR. At the protein level, MRP-14 2E5 KO mice expressed neither MRP-14, nor MRP-8. As was found with the MRP-8 KO mice, no significant differences in cell populations in either the blood or bone marrow compartments were observed between MRP-14 WT and KO mice.

6.4.2 Problems with generating MRP-8 KO and MRP-14 KO mice

Distinct problems were encountered in the generation of MRP-8 KO and MRP-14 KO mice. The main problem encountered in the production of both 5FD10 and 3C11 MRP-8 KO mice was the correct genotyping of animals, as the PCR based genotyping assay produced incorrect results. The problem was that WT mice were typed as WT or HET and KO mice were always typed as HET. This caused a delay of approximately 6 months in the typing of the first MRP-8 KO mice and the problem was only rectified once Southern hybridisations of the samples had been performed.

PCR genotyping was relied upon because HET ES cells, 129 DNA (the strain that gene targeting is performed in) and the KOC (which mimics the KO mouse by providing only a mutant gene as a template), produced the expected PCR banding patterns for their respective genotypes. Therefore, it was considered appropriate to use the PCR assay to genotype mice in the breeding programs.

One possible cause of this result could have been related to the PCR genotyping assay being based within the construct, rather than on the construct but with reference to the position of the construct within the genome (as is the case for Southern hybridisations). In practice, this means that in a HET, as well as the targeted replacement event that was screened for in ES cells, if another integration of another copy of the MRP-8 KOC had occurred randomly in the genome, this would be picked up by the PCR assay as an additional source of a mutant band. This would explain the typing of WT mice as HET mice if the WT mice had inherited a non-targeted construct. However, this does not explain the mis-typing of KO mice as HET mice. The source of this mis-typing is unknown.

The main problem encountered in the generation of MRP-14 mice was one of non-transmission of the mutant gene from the chimera, to progeny, in order to generate the first heterozygous mice. This problem delayed the project by 20 months. This non-
transmission, described in detail in section 6.3.2 (page 267), was characterised by 3 chimeric lines that did not transmit any coat colour and 2 chimeric lines, 12H and 12G that did transmit coat colour but not the targeted MRP-14 gene.

The source of this problem was thought not to be the ES cell line used to create the chimeras, as this had been used to successfully transmit targeted gene mutations in various knockout projects (including MRP-8). The problem of non-transmission was only resolved with a second transfection, using a new cell line, followed by the creation of new chimeras. It was these new chimeras, from clones 2E5 and 2G1 that transmitted the targeted MRP-14 genes, which finally allowed transmission and identification of the first HET mice.

6.4.3 Gross phenotypes and breeding characteristics of MRP-8 KO and MRP-14 KO mice

Neither MRP-8 nor MRP-14 KO mice displayed a gross phenotype as measured by apparent vigour, health or adult mass. MRP-8 KO mice were found not to succumb to any particular diseases or ailments more often to be as long-lived as their WT counterparts (observations up to approximately 1 year of age). A comparison with MRP-14 KO mice was not possible due to their relatively late production within the time available.

When a HET versus HET pair breeds, according to Mendelian genetics, a 1:2:1 ratio of WT:HET:KO is expected (if the gene in question is carried on an autosome). This ratio was not found for either of the MRP-8 clones. 5FD10 produced a ratio of 1:0.73:0.4 and 3C11 produced a ratio of 1:0.76:0.59. In contrast, the MRP-14 clone, 2E5, produced an almost perfect ratio of 1:1.56:1.08.

One possible reason for the perturbation of the expected ratio when breeding MRP-8 mice may be a reabsorption event. If MRP-8 was necessary for development, then a reduced number of KO mice might be expected; in this case, such mice that were born may be sickly. In addition, an increased number of HET mice would also be expected, shifting the ratio of WT:HET:KO towards 1:2:0. In fact, KO mice appear to have as much vigour as their HET and WT littermates and no increase in the number of HET mice born was observed. Indeed, as well as a decreased number of KO mice born, there was also a decreased number of HET mice born, which would not be predicted from such a model. When comparing the number of HET and KO mice born, the ratio of
HET:KO was almost 2:1, which would suggest no role for MRP-8 in development. Also, litter sizes appear to be similar for MRP-8 and MRP-14 HET versus HET intercrosses (MRP-14 is known not to be required for development). Furthermore, MRP-8 KO versus KO pairs breed normally, producing normal litter sizes. Taken together, these lines of evidence suggest that a reabsorption event is not occurring and that MRP-8 is not required for, or is redundant in, development.

An alternative explanation of the disturbed Mendelian ratio would be an over-representation of WT mice in the MRP-8 HET versus HET litters. This could be due to a compromised sperm function of those sperm carrying the mutant MRP-8 gene. In a HET versus HET pairing, this would be expected to decrease the number of KO mice but not HET mice. To decrease the numbers of HET and KO mice, rather than just decreasing the number of KO mice, a compromised ova production or function would also have to be considered. In this case, perhaps MRP-8 may be involved in embryo implantation. Interestingly, any decrease in zygote function of those zygotes carrying mutant MRP-8 genes appears to only be in relation to WT zygotes as KO versus KO pairs breed normally and produce normal litter sizes. It maybe of interest for a future investigator to measure the relative motility of MRP-8 WT and KO sperm, or the role of MRP-8 in embryo implantation.

### 6.4.4 Relative mRNA levels in MRP-8 KO and MRP-14 KO mice

RT-PCR was the technique chosen to assay MRP-8 and MRP-14 mRNA levels in both MRP-8 and MRP-14 transgenic mice. In the case of the MRP-8 transgenic mice, the RT-PCR results were verified and quantified by hybridising the RT-PCR reactions with specific probes. This ensured that non-specific products, produced in the PCR reaction, were not quantitated in the final analysis of mRNA expression levels. In the case of MRP-14 transgenic mice, the RT-PCR results were not verified by hybridisation but by using another set of primers that have been found not to generate non-specific products. Also, it was not as important to know the MRP-14 mRNA levels in MRP-14 KO mice, as by that point, MRP-14 protein levels had been shown to be zero in the KO animals by Western blot analysis.

MRP-8 KO animals express 9% of WT levels of MRP-8 mRNA and 20% of WT levels of MRP-14 mRNA. The reason for any expression of MRP-8 mRNA in the KO animals is not clear. An exon-interruption cassette was used to target the MRP-8
gene and the correct targeting of this construct was confirmed by Southern hybridisation. Also, MRP-8 KO mice did not express any MRP-8 protein, as measured by Western blot analysis. Two biological hypotheses would explain this set of results. Firstly, the MRP-8 mRNA found in the KO mice was not functional. That is, it could not produce protein. Secondly, that MRP-8 protein was being produced from the MRP-8 mRNA, but in such low levels that it could not be detected by Western blot analysis.

Both biological hypotheses require transcription of MRP-8 mRNA. Assuming that both copies of the endogenous gene have been replaced in a KO animal, MRP-8 mRNA production still needs to occur. A possible mechanism for this could be if transcription initiated in exon 1, as normal, but then read through the NEO cassette (present in an antisense orientation; see section 5.2.3, page 200) and the rest of the MRP-8 gene normally. The Neo mRNA may then be spliced out from the MRP-8-Neo mRNA and this would produce the mRNA species identified in the RT-PCR reaction.

The first hypothesis, of a non-functional mRNA, then further requires that the splicing event generates a non-functional mRNA molecule, perhaps by deletion of the start codon. In contrast, the second hypothesis further requires that such a splicing event produces a functional mRNA molecule, by complete removal of the Neo cassette. The full MRP-8-Neo transcription event, followed by splice removal of the Neo mRNA may be considerably rarer than a normal MRP-8 transcription event, possible because the majority of hybrid transcripts are unstable and degraded. This could explain the decreased MRP-8 mRNA levels detected. Interestingly, other RT-PCR investigations have shown that the Neo cassette mRNA does not exist as part of a stable hybrid MRP-8-Neo mRNA species (data not shown). This may suggest that the hybrid molecule, if it is transcribed, is unstable.

One artefactual hypothesis, which is similar to the non-functional mRNA hypothesis, would also explain these results. Given that the cDNA primers used to assay the amount of MRP-8 mRNA present contain the start codon and 6 other codons of MRP-8 mRNA sequence, it is possible that if the MRP-8 mRNA species identified in the assay was missing a start codon, that the forward primer would not bind with 100% efficiency at the annealing temperature used in the assay. This may result in decreased annealing and decreased product formation; this may explain the RT-PCR results. As to the nature of the template, assuming that the MRP-8-Neo mRNA hybrid
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is not stable, the template may be an incorrectly spliced MRP-8 mRNA, produced from such a MRP-8-Neo mRNA hybrid. This may be produced in as large amounts as MRP-8 mRNA is produced in WT animals. As the forward primer does not bind correctly, this could then produce the RT-PCR results seen.

To resolve this issue, two things should be done. The first would be to repeat the results with a Northern hybridisation. This would rule out any RT-PCR artefacts that have not been considered so far. The second would be to clone and sequence the DNA species generated in the RT-PCR analysis. This would show whether the molecule was a functional mRNA species, or not.

MRP-14 KO mice were found to express no MRP-14 mRNA, when using the cDNA primers in a RT-PCR reaction. In contrast, when using exon 3 specific primers, 10-20% of WT levels of that portion of the mRNA species were detected. MRP-8 mRNA levels did not seem to be affected by the abrogation of MRP-14 mRNA levels. These results must be tempered by the fact that a hybridisation was not performed to quantitate the reaction and to ensure that the bands visualised on the gel were true amplifications of the specific mRNA molecules.

As they stand, these results suggest that no full length MRP-14 mRNA is being produced in the KO animals. This was expected given that there is a stop codon at the beginning of the interruption cassette (see section 5.3.3, page 212). They also suggest that a relatively small amount of exon 3 is being transcribed into a stable mRNA species. It is unlikely that this mRNA species is being translated into protein as there are no reported splice variants of MRP-14 (140).

The impact of MRP-8 mRNA abrogation by gene targeting on MRP-14 mRNA levels will be discussed in the next section.

6.4.5 MRP-8 and MRP-14 protein expression in MRP-8 KO and MRP-14 KO mice

Targeting the MRP-8 gene was found to abolish MRP-8 expression and decrease MRP-14 expression to 50% of WT levels, as measured by Western blot analysis. In contrast, targeting the MRP-14 gene was found to abolish both MRP-8 and MRP-14 protein expression, by Western blot analysis. Clearly, these 2 sets of data are slightly but importantly different.

Data gained from immunoaffinity purification of bone marrow lysates with a monoclonal anti-MRP-14 antibody suggested that MRP-8 is complexed to MRP-14, in
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\textit{vivo}, as has been previously reported (129). Therefore it is not unexpected that the relative protein expression levels of either MRP-8 or MRP-14 would impact, one upon the other.

The relationship that defines how the molecules are expressed, relatively and absolutely, is not simple. It seems that MRP-8 is not required for MRP-14 expression. It is however, associated, in as much as lack of MRP-8 protein diminishes MRP-14 protein expression. On the other hand, MRP-14 is absolutely required for MRP-8 expression. MRP-8 protein cannot be expressed without MRP-14 protein. In summary, MRP-14 but not MRP-8 can be expressed alone in so far as leukocyte expression is concerned.

Analysis of the relative mRNA expression of MRP-8 and MRP-14 molecules within the MRP-8 and MRP-14 KO models adds another layer of complexity to the relationship. Although MRP-14 protein expression is absolutely required for MRP-8 protein expression in the MRP-14 KO, the levels of MRP-8 mRNA appear to be the same in the KO as in the WT. This suggests that the level of control is at the translational, not at the transcriptional level.

In the MRP-8 KO, MRP-14 protein expression levels are 50% of those in the WT. This appears to be due to transcriptional regulation as MRP-14 mRNA levels are down-regulated to 20% of WT levels. In contrast to the MRP-14 KO where MRP-8 protein expression is regulated at the translational level, in the MRP-8 KO, MRP-14 expression appears to be regulated at the transcriptional level.

As has been mentioned previously, one problem with the above hypothesis is that the MRP-14 KO RT-PCR results, which suggested the relative MRP-8 mRNA levels, are tempered because they were not hybridised. This may mean that in the MRP-14 KO, actual (gel followed by hybridisation analysis), rather than perceived (gel alone) MRP-8 mRNA levels may actually be decreased and not unchanged (as suggested above). This would then suggest a common, transcriptional regulation of protein expression by both MRP-8 and MRP-14 on the partner molecule (MRP-14 and MRP-8, respectively).

Whether the protein expression relationship between MRP-8 and MRP-14 turns out to be regulated at the transcriptional or translational level, it is clear that the relationship is not equal. MRP-8 protein expression is associated with MRP-14 protein expression. The expression of some MRP-8 protein allows greater expression levels of
MRP-14 protein than the expression levels of MRP-14 protein found in the absence of MRP-8 protein. In contrast, MRP-14 is absolutely required for MRP-8 expression.

This pattern may suggest one or both of the following hypotheses. Firstly, that MRP-8 is more unstable as a protein than MRP-14 and so it cannot exist without its partner, which acts to stabilise it. Attempts to express recombinant MRP-8 and MRP-14 would support this instability hypothesis. Secondly, MRP-8 is a potent chemotactic agent, \textit{in vitro} (137) and \textit{in vivo} (138) whereas MRP-14 has only been shown to be chemotactic \textit{in vivo}. There is now good evidence for the existence of a murine MRP-8/14 heterodimer (129). In humans, the heterodimer is so far considered to be functionally inactive, whereas the MRP-14 subunit induces adhesion of neutrophils (198). If the mouse heterodimer is also functionally inactive then it is possible that MRP-8 is too potent a chemoattractant to exist in isolation from the natural functional inhibition or “brake” of MRP-14. This would then further imply that MRP-14 is less functionally important than MRP-8 as a chemoattractant, as MRP-14 does exist without the natural functional inhibition of MRP-8.

6.4.6 Examination of cell population changes in MRP-8 KO and MRP-14 KO mice

Various changes were seen in the relative cellularity between WT and KO mice, for both MRP-8 and MRP-14 transgenic mice. That is, when comparing a WT to KO mouse in a FACS assay, various changes were seen in the relative percentage of cell types, within blood or bone marrow cell populations. However, these changes were variable between experiments and therefore did not translate to visible changes after averaging results.

As MRP-8 and MRP-14 are so abundantly expressed within myeloid cells, particular attention was directed to the relative abundance and differentiation stage of neutrophils. This was done using the antibodies 7/4, which reacts with a polymorphic 40kDa antigen expressed on 129J and C57/BL6 derived neutrophils, and Gr-1, which reacts with a set of lineage restricted antigens, most prevalently expressed on more differentiated granulocytes. These analyses suggested that MRP-8 KO and MRP-14 KO mice may have had an increased number of neutrophils (as marked by 7/4), which were well differentiated (as marked by Gr-1) than WT mice, both in the blood and the bone marrow (data not shown). As previously described, these differences were not significant when averaged. Further FACS experiments and experiments on isolated neutrophils may prove whether this trend is real. If the trend is real, it may suggest that...
MRP-8 and MRP-14 are not involved in differentiation and are simply differentiation markers.

One crucial question has not been addressed by this work. Given the abundance of MRP-8 and MRP-14 in myeloid cells, and given that these cells appear to be present in MRP-8 and MRP-14 KO animals in apparently normal amounts, what is replacing MRP-8 and MRP-14 in these cells to make up the large protein deficit? It would be of considerable interest to identify candidate replacement proteins. One way a future investigator might address this issue could be through the use of 2D-protein gel analysis.

6.4.7 Future directions

This work has only begun to uncover the roles of MRP-8 and MRP-14 in vivo. Further basic phenotyping studies, such as examining possible cell population changes are still to be completed for both MRP-8 and MRP-14 transgenic mice. Indeed, confirmation of the successful abrogation of MRP-14 expression in mice from the 2G1 clone has yet to be obtained.

If this investigator were to continue the study, priority investigations would include the following. For both MRP-8 and MRP-14 transgenic mice, isolated neutrophils from the blood and bone marrow would allow studies on gross and fine structure with basic light and electron microscopy. This would also allow 2D protein gel electrophoresis, to identify the protein(s) that replace the abrogated MRP protein(s). These isolated cells could then be used to evaluate standard neutrophil functions such as calcium flux, chemotaxis, phagocytosis, oxidative burst and granule release. A particularly elegant method to assess phagocytosis has been described which uses FITC-coated yeast to facilitate assessment of phagocytosis (23, 217).

A particularly interesting area to address would be the potential role of MRP-8 and MRP-14 in inflammation, with reference to myeloid cells. Preliminary results using MRP-8 transgenic mice have suggested a role for MRP-8 in facilitating the early arrival (<4h) into the peritoneum after inflammatory challenge. Various models could be used to assess potential roles of the MRP proteins, such as the delayed type hypersensitivity DNFB ear thickness model, the air pouch model and the peritoneal thioglycollate model.

It would also be of interest to discover the role of MRP-8 and MRP-14 in infection models. These models can be traumatic for the animals so perhaps the best
way to address the problem would be assess the phagocytosis and killing of bacteria ex vivo, adapting the methods for the assessment of phagocytosis, described above.

A recent report has shown that EN-RAGE, a member of the S100A family that is completely homologous to bovine MRP-6 is involved in the induction of the inflammatory process by ligation of RAGE (receptor for advanced glycation end-products) (102). Antibodies to this protein block the induction of inflammation in mice and recognise a protein in mouse plasma, released after LPS infusion. This protein may be murine MRP-6. As a MRP family member, expression of this protein may be affected by the manipulation of MRP-8 and MRP-14 expression. It would be of considerable interest to evaluate whether murine MRP-6 exists and whether its protein expression is affected by MRP-8 and MRP-14. Also, it would be interesting to discover whether any perturbations in inflammation in the MRP-8 or MRP-14 KO mice were mediated by RAGE.

Future work will mainly concentrate on analysing the phenotype of the MRP-8 and MRP-14 transgenic animals that have been generated in the course of this work, especially in functional studies. It will be important to take into account that when studying MRP-8 transgenic animals, the study is not assessing the role of MRP-8 in a given process, but rather the role of MRP-8 and half the WT levels of MRP-14. In contrast, when studying the MRP-14 animals, the study is assessing the role of MRP-8 and MRP-14 together, in a given process. These differences will be in addition to the roles played by the adaptation of the whole in vivo system to the removal of MRP-8 and MRP-14, such as the increase in some protein(s) within myeloid cells to replace MRP-8 and MRP-14. This may make the original aim of the project, the functional separation of roles for MRP-8 and MRP-14 in vivo, rather more difficult than originally envisaged. However, if the work proposed above is completed, perhaps the role of MRP-8 and MRP-14 in neutrophil function and general inflammation will finally be elucidated.
7. Discussion and Conclusions

This short chapter will draw together the findings of this thesis and from this suggest future directions for the work. Future directions will be incorporated in the general discussion and summarised at the end of the chapter.

The aims of this thesis were to characterise the function of MRP-8 and MRP-14 in myeloid cells, particularly neutrophils and also in the immune response, to challenges which elicit myeloid cells. The methods used to do this were by gene inactivation via gene targeting and the production and characterisation of recombinant protein.

The main findings of this thesis have been the demonstration of a functional role for MRP-14 in leukocyte migration, using recombinant MRP-14. Other main findings resulted from gene targeting experiments which have shown that MRP-8 and MRP-14 are not necessary for mouse or neutrophil development.

The demonstration of a role for MRP-14 in leukocyte migration was achieved using recombinant MRP-14, produced in E.coli. It was hoped to also produce recombinant MRP-8, in order to reproduce and expand the findings of direct chemotaxis, reported by another group (137). Various attempts were made to produce both recombinant MRP-8 and MRP-14 but recombinant MRP-8 production was not successful (see chapter 3). Given the findings in this thesis, that MRP-14 is involved in leukocyte migration, it would be particularly interesting to produce MRP-8 and study the role of the MRP-8/14 complex in leukocyte migration and general function. To achieve this, a future investigator may wish to generate GST-MRP-8, as has been described (113).

Both in vitro and in vivo profiling of MRP-14 function was performed. In vitro, MRP-14 was found to stimulate peripheral blood mononuclear cell, peripheral neutrophil, thioglycollate-elicited neutrophil and WEHI 265.1 monocytoid cell line, adhesion to fibrinogen. The cellular mediator of this interaction was not elucidated but was thought to be Mac-1 due to the ligand used and also because of the demonstration that human MRP-14 induced human neutrophil binding to human fibrinogen using the β2-integrin, Mac-1 (198).

Various attempts were made to prove the involvement of Mac-1 using the Mac-1-blocking antibodies, M1/70 and 5C6. However, blocking of MRP-14 induced adhesion was not seen with these antibodies. There is another candidate adhesion molecule that could mediate the MRP-14 induced cellular adhesion interaction to
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fibrinogen, CD11c/CD18 (160). A future investigator may wish to source blocking antibodies or knockout cells (from CD11c/CD18 knockout mice) in order to investigate the role of CD11c/CD18 in this process.

Further in vitro studies using thioglycollate-elicited leukocytes showed that MRP-14 did not cause superoxide burst, calcium flux or chemotaxis, at any concentrations tested. In contrast, leukocytes derived from peripheral blood did undergo calcium flux (at 0.1μM), but not superoxide burst. Given the dichotomy in the response of peripheral and thioglycollate-elicited cells to MRP-14 it was possible that a dichotomy could also exist in the chemotaxis response. This was investigated but an answer was not obtained. Therefore, it remains to be determined if MRP-14 causes chemotaxis in peripheral leukocytes. If chemotaxis was proven, this would make MRP-14 a novel chemoattractant that acts solely on peripheral, resting cells. This is the main question that remains to be answered about the function of MRP-14 in vitro and could be addressed with peripheral leukocytes used in the thioglycollate-elicited leukocyte chemotaxis assay (see section 4.3.3.1, page 146).

In vivo studies in the mouse air pouch model showed that MRP-14 was a chemoattractant, recruiting 70% neutrophils and 30% monocytes with an optimal dose of 50μg at a maximally active time of 6 hours. MRP-14 was found to act more quickly than the indirectly acting chemoattractant LPS, which had a maximal response at 12h and more slowly than the directly acting chemoattractant, MIP-2. Taken together with the data showing that MRP-14 was not chemotactic for thioglycollate-elicited leukocytes, it was suggested that MRP-14 was acting indirectly, through the release of an unknown mediator(s), from unidentified cell(s), to recruit leukocytes to the air pouch. In the event of MRP-14 being found not to be chemotactic for peripheral leukocytes, further work might address the source and the agent(s) responsible for the recruitment of leukocytes to the air pouch. This could be achieved by the RT-PCR assessment of differing transcript levels in air pouch tissue.

Other investigation of MRP-8 and MRP-14 function was via the generation and analysis of MRP-8 and MRP-14 KO mice. This produced the other main findings of this thesis, that MRP-8-deficient and MRP-14-deficient mice developed normally and had normal neutrophil levels. This suggested that neither MRP-8 nor MRP-14 were necessary for either normal murine development or for normal neutrophil development.
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Whilst MRP-8-KO mice had no MRP-8 and 50% MRP-8-WT levels of MRP-14 protein, MRP-14-KO mice had neither MRP-8 nor MRP-14 protein. This suggested that MRP-14 but not MRP-8 can be expressed alone. Furthermore, this suggested that whilst MRP-8 is permissive for MRP-14 expression, MRP-14 is absolutely required for MRP-8 expression. Taken together, these data support a situation in vivo, in which a protein complex of MRP-8 and MRP-14 exists. Given that both molecules are chemoattractants, this complex could be hypothesised to be functionally inactive. This would explain how molecules with such potent cellular effects could be safely expressed at such high levels.

MRP-8 is a direct chemoattractant for neutrophils, optimally active at $10^{13}$M (137) whereas MRP-14 is an indirect chemoattractant for neutrophils and monocytes, active in vivo at a dose concentration of 1.8 μM. Although the data are difficult to compare because they are from different systems, it may be hypothesised that MRP-8 is a more potent chemoattractant than MRP-14. This hypothesis correlates well with the data from the MRP-8 KO and MRP-14 KO mice showing that MRP-14 but not MRP-8 can be expressed alone.

Only very basic phenotyping has been performed with the MRP-8-KO and MRP-14-KO mice. To date, the data suggests that there was no significant difference in either relative cellularity or in the number, size or shape of peripheral or bone marrow neutrophils in either MRP-8-KO or MRP-14-KO mice. This data is preliminary and requires further confirmation from a future investigator.

Preliminary investigation of the phenotype of MRP-8-KO but not MRP-14-KO mice in inflammatory models was performed. MRP-8-KO mice are less able than MRP-8-WT mice to recruit leukocytes to the peritoneum at 4 hours although the effect disappears at 24 hours. This suggests a role for MRP-8 in leukocyte migration. What the basis of this effect is, whether it is the lack of MRP-8 protein causing a general defect in neutrophil function, whether calcium signalling is impaired in the neutrophil or whether integrin function (perhaps activation) is impaired remains to be seen. Clearly, this is the beginning of the inflammatory phenotyping and a future investigator would wish to extend these investigations to other models of inflammation, such as the air pouch and also incorporate MRP-14-KO mice. Also, no in vitro functional analysis of the myeloid cell function of MRP-8-KO and MRP-14-KO mice has been performed. Examples of the types of studies that could be performed include analysis of calcium
signalling by measurement of calcium flux, measurement of neutrophil effector functions such as phagocytosis, superoxide burst and granule release, adhesion, chemotaxis and transmigration assays.

A future approach that may be considered to further elucidate the function of MRP-8 and MRP-14 would be to re-target the MRP-8 and MRP-14 genes using the Cre-loxP system. One possible approach would be to use a neutrophil specific promoter, such as elastase, to drive Cre recombinase so that only neutrophil, and not specialised epithelia protein expression would be ablated. Alternatively, the same promoter, or another tissue specific promoter could also be coupled up to a tetracycline-sensitive cassette so that the temporal removal of either MRP-8 or MRP-14 could be decided by the investigator and not by the time at which the promoter driving Cre became active. Also, the use of such a system would obviate the concerns about traditional knockout mice, that the mice are adaptive organisms, formed from conception without the protein of interest.

7.1 Summary of suggestions for future work

- Produce recombinant GST-MRP-8 to allow reproduction and expansion of previously published work (137).
- Investigate role of CD11c/CD18 in mediation of MRP-14 induced leukocyte adhesion to fibrinogen using blocking antibodies and/or knockout cells.
- Investigate whether MRP-14 is chemotactic for peripheral leukocytes in the thioglycollate-leukocyte chemotaxis system (24 well 3μm Transwell system).
- If MRP-14 is not chemotactic, investigate the cell type and the agent(s) responsible for mediating the in vivo chemoattractant response of MRP-14 with RT-PCR profiling of air pouch tissue.
- Full bone marrow and peripheral blood cell type characterisation in both MRP-8-KO and MRP-14-KO mice.
- Extension of MRP-8-KO thioglycollate-elicited peritonitis data, inclusion of MRP-14-KO mice and extension of inflammatory modelling to other models such as the air pouch.
- In vitro analysis of neutrophil function in the MRP-8-KO and MRP-14-KO mice. Example studies: - calcium flux, phagocytosis, superoxide burst, granule release, adhesion, chemotaxis and transmigration.
• Re-target the MRP-8 and MRP-14 genes using the Cre-loxP system. Target the Cre recombinase using tissue specific promoters and/or drug-on/off-tissue specific promoters.

7.2 Conclusions

In conclusion, MRP-14 was found to be an indirectly acting chemoattractant for neutrophils and monocytes that did not cause chemotaxis or cellular activation of pre-migrated cells, in terms of activation of the superoxide burst or induction of calcium flux. However MRP-14 did cause calcium flux but not superoxide burst in naïve peripheral blood leukocytes. Some work remains to be completed to fully characterise the function of this molecule, either as an indirectly acting chemoattractant or as a directly acting chemoattractant, specific for naïve, peripheral leukocytes.

Gene targeting revealed that, intracellularly, MRP-14 protein is necessary for the expression of MRP-8 protein but that expression of MRP-8 protein is not necessary for MRP-14 expression. Ablation of either gene product, MRP-8 in the MRP-8-KO or both MRP-8 and MRP-14 in the MRP-14-KO, did not affect normal mouse development or neutrophil development. Lack of MRP-8 affects migration into the peritoneum at the 4 hour timepoint.

This work will form the basis for some fascinating future insights into the function of the two myeloid cell calcium-binding proteins, MRP-8 and MRP-14.
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