A ROLE FOR S100 PROTEINS, MRP-8 AND MRP-14 IN LEUKOCYTE ADHESION

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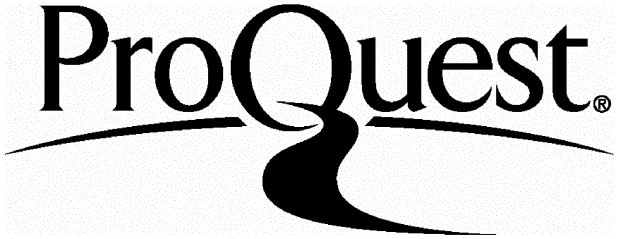

ABSTRACT

Migration inhibitory factor related protein (MRP)-8 and MRP-14 are S100 proteins primarily expressed by circulating human monocytes and neutrophils. *In vivo* MRP-8 and MRP-14 exist as a heterodimer (MRP-8/14) and represent ~40% of neutrophil cytosolic protein (~1% in monocytes). MRP-8/14 is also known to be expressed on the surface of neutrophils and monocytes and MRP-8 and MRP-14 have been detected in the extracellular environment. Despite their relative abundance and known expression patterns, no definitive function for the human proteins existed, to date. Previous results from our laboratory show extracellular localisation of MRP-8/14 on endothelium adjacent to marginating neutrophils and monocytes. This observation stimulated an investigation into the role of MRP-8/14 in neutrophil adhesion and transmigration. Initial experiments using purified native material indicated that MRP-8/14 proteins were unable to stimulate neutrophil adhesion. Further analysis, with individual recombinant proteins, revealed that only MRP-14 could induce neutrophil adhesion, mediated by the β_2 integrin Mac-1. MRP-8 did not induce any adhesion, but was able to inhibit the adhesion induced by MRP-14 through the formation of the heterodimer. MRP-14 was not able to induce subsequent transmigration of neutrophils, nor was it able to activate other neutrophil effector functions, such as changes in cell surface receptor expression, exocytosis, respiratory burst or Ca^{2+} flux. Instead, MRP-14 binds to a discrete G protein-coupled receptor on the neutrophils generating signals leading only to the activation of Mac-1.

MRP-14 also bound to T lymphocytes and similarly induced Mac-1 activation. In addition, MRP-14 was also able to activate β_1 integrin adhesion to fibronectin by these cells. Thus, it appears that MRP-14 by binding to its receptor is able to directly activate integrins allowing leukocyte adhesion. This results represent the first description of a function for MRP-8 and MRP-14 and suggest MRP-14 as an *in vivo* candidate of integrin activation resulting in leukocyte adhesion to endothelium.

ACKNOWLEDGMENTS

I know this is the first part of a thesis anyone reads....and, well, what can I say?

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For those that are still left, I guess I should thank you for putting up with having a loud Australian in the lab. I've really enjoyed my time in the MacLab and I'm sure not many people have this much fun at work. It's been great! Thanks to Richard May and Mat Robinson (for their cheekiness), Birgit Leitinger, Paula Stanley, Gleb Ivanov, Philippe Tessier for making up the atmosphere in the MacLab. A special thanks to Ali McDowall and Jo Porter for lots of late night and weekend chats. To Ali, also I thank you for all your help over the years and for nights out at The Crusting Pipe and Maxwell's.
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And finally, thanks must go to my family who reluctantly let me travel so far from home but have supported me completely. This is for you.

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ABBREVIATIONS

ADP	adenosine diphosphate,
APC	antigen presenting cell,
APS	ammonium persulphate
ATP	adenosine triphosphate,
BCECF/AM	(2',7-bis(carboxyethyl)-5(6')-carboxyfluorescein pentaacetoxymethyl) ester),
BSA	bovine serum albumin,
C5a	anaphylactic product of fifth component of complement,
CD	cluster of differentiation,
cDNA	complementary deoxyribonucleic acid
CGL	chronic granulomatous leukaemia
CK	casein kinase,
CO_2	carbon dioxide
CP-10	chemotactic protein 10kDa,
CR	complement receptor,
CS I	connecting segment I
DHR	dihydrorhodamine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid disodium salt
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (b-aminoethyl ether) N,N,N'N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ESL-1	E-selectin ligand-1
F/P	fluorescence/protein ratio
F-actin	filamentous-actin
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
FcγR	receptor for the Fc portion of IgG
FCS	foetal calf serum

FITC	fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
FPLC	fast protein liquid chromatography
FURA-2/AM	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethyl) ester
GAG	glycosaminoglycan
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GPI	glycosyl-phosphatidyl inositol
GTP	guanine nucleotide triphosphate
h	hour
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HEV	high endothelial venules
HMWK	high molecular weight kininogen
HPLC	high performance liquid chromatography
IAP	integrin-associated protein
iC3b	inactivated third component of complement
ICAM	intercellular adhesion molecule
ICRF	Imperial Cancer Research Fund
Ig	immunoglobulin
IgSF	immunoglobulin supergene family
IL	interleukin
ILK	integrin-linked kinase
IMF-1	integrin modulating factor-1
IP_3	inositol triphosphate
kDa	kilodaltons
LAD	leukocyte adhesion deficiency
LAL	leukocyte adhesion lipid
LFA	leukocyte function-associated antigen
LIBS	ligand induced binding site
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MEK	MAPK/ERK kinase

MHC	major histocompatibility complex
MIDAS	metal ion dependent adhesion site
MIF	macrophage migration inhibitory factor
min	minute
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
MRP	MIF-related protein/myeloid-related protein
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
NIF	neutrophil immobilising factor
OD	optical density
OPD	o-phenylenediamine dihydrochloride
nm	nanometre
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PECAM-1	platelet-endothelial cell adhesion molecule
PHA	phytohaemagglutinin
PI 3-kinase	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol bisphosphate
PIP_3	phosphatidylinositol trisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol-12-myristate-13 acetate
PMSF	phenyl-methyl-sulphonyl-fluoride
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	regulated on activation normal T cell expressed and secreted
RBC	red blood cell
RT	room temperature
SDS	sodium dodecyl sulphate
sICAM	soluble ICAM
sLe^a	sialyl $Lewis^a$ antigen
sLe^x	sialyl $Lewis^x$ antigen
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	tumour necrosis factor
uPAR	urokinase receptor

VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen
vWF	von Willebrand factor
$[Ca^{2+}]_i$	intracellular calcium

INTRODUCTION

1.1 LEUKOCYTES ADHESION RECEPTORS IN THE IMMUNE SYSTEM

Leukocytes, or white blood cells, are motile cells which primarily circulate in the vascular system. The majority of leukocytes are either the mononuclear monocytes and lymphocytes, or the polymorphonuclear neutrophils, eosinophils and basophils (granulocytes). Neutrophils and monocytes (and macrophages) are also described as myeloid cells based on their development from myeloid cell stem cells in the bone marrow. In general, myeloid cells are part of the innate immune system and as such are responsible for the direct killing of pathogens by engulfing them. This process is termed phagocytosis and is dependent on two types of cell surface receptors; complement receptors and immunoglobulin (Ig) receptors. On myeloid cells, complement receptor (CR) 1, CR3 and CR4 are responsible for binding pathogens which have been opsonised with complement products, C3b and iC3b; whereas, Ig receptors bind the Fc portion of Ig bound to antigen (Silverstein et al., 1989). Synergistic activation of these receptors, results in the successful endocytosis of pathogens into vesicles known as phagosomes. Endocytosed pathogens are then lysed by the release of granular enzymes and oxidative metabolites into the phagosome. The complement receptors CR3 and CR4 have since been identified as members of the integrin family of adhesion molecules and are also referred to as Mac-1 and p150,95, respectively (see section 1.3.2.1).

The lymphoid cells, made up primarily of T and B lymphocytes provide the body with a specialised surveillance mechanism for foreign antigen as well as immunological memory. T and B lymphocytes are required to patrol the body for foreign antigen by recirculating from the blood into tissues, returning to the blood via the lymph nodes. T lymphocytes recognise antigen through their T cell receptor (TCR) complexed to CD3 (TCR/CD3) and in the context of major histocompatibility complex (MHC) on the surface of antigen presenting cells (APC) or target cells. T cells can be further divided into two subtypes based on their expression of either CD4 or CD8, which determines their effector function. CD4+ T (or T helper) cells recognise processed peptides from exogenous antigen presented by MHC class II molecules on antigen-presenting cells. Signals from the TCR/CD3 complex and CD4 lead to T cell clonal proliferation and

'help' in the form of cytokine production. CD8+ T cells recognise viral antigen products in the context of MHC class I molecules, and co-stimulatory signals generated from ligation of TCR/CD3 and CD8 result in the production of cytotoxic granules which are able to directly lyse the virally-infected cell. Another family of molecules are critical to the interaction with antigen-presenting cells and these are termed lymphocyte function-associated antigens (LFA)-1, -2 (CD2) and -3 (reviewed in (Springer, 1990)). The LFAs have since been shown to be members of two families of adhesion molecules, the integrins (LFA-1; see section 1.3.2.1) and the immunoglobulin supergene family (IgSF; LFA-2 and -3).

The integrin family members, CR3 (Mac-1) and LFA-1, involved in the identification and destruction of pathogens, are also involved in the migration of myeloid cells from the blood stream into the pathogen-infected tissues. LFA-1 is also central to the successful lymphocyte recirculation through blood and lymph nodes, and the response of lymphocytes to inflammation, further emphasising the importance of these adhesion receptors in the immune system.

1.2 LEUKOCYTE EMIGRATION AND RECIRCULATION

Whether or not a particular cell type is recruited to an inflammatory site depends on many factors, including the nature of the inflammatory stimuli and the type of tissue affected. Thus, there is great potential for cellular and tissue diversity in the response of leukocytes to inflammation. Despite this, a general sequence of receptor-mediated events appears to control the response to inflammation. This sequence of events is termed the "leukocyte adhesion cascade" and, for leukocytes in general, involves four distinct steps: "rolling", activation, firm adhesion and transmigration, mediated by three known families of adhesion molecules, the selectins, the integrins and the IgSF. The "cascade" for the individual cell types, neutrophils, monocytes and lymphocytes, is summarised in **Figure 1.1**. This sequence of adhesion receptor interactions is also applicable to lymphocyte transmigration necessary for recirculation.

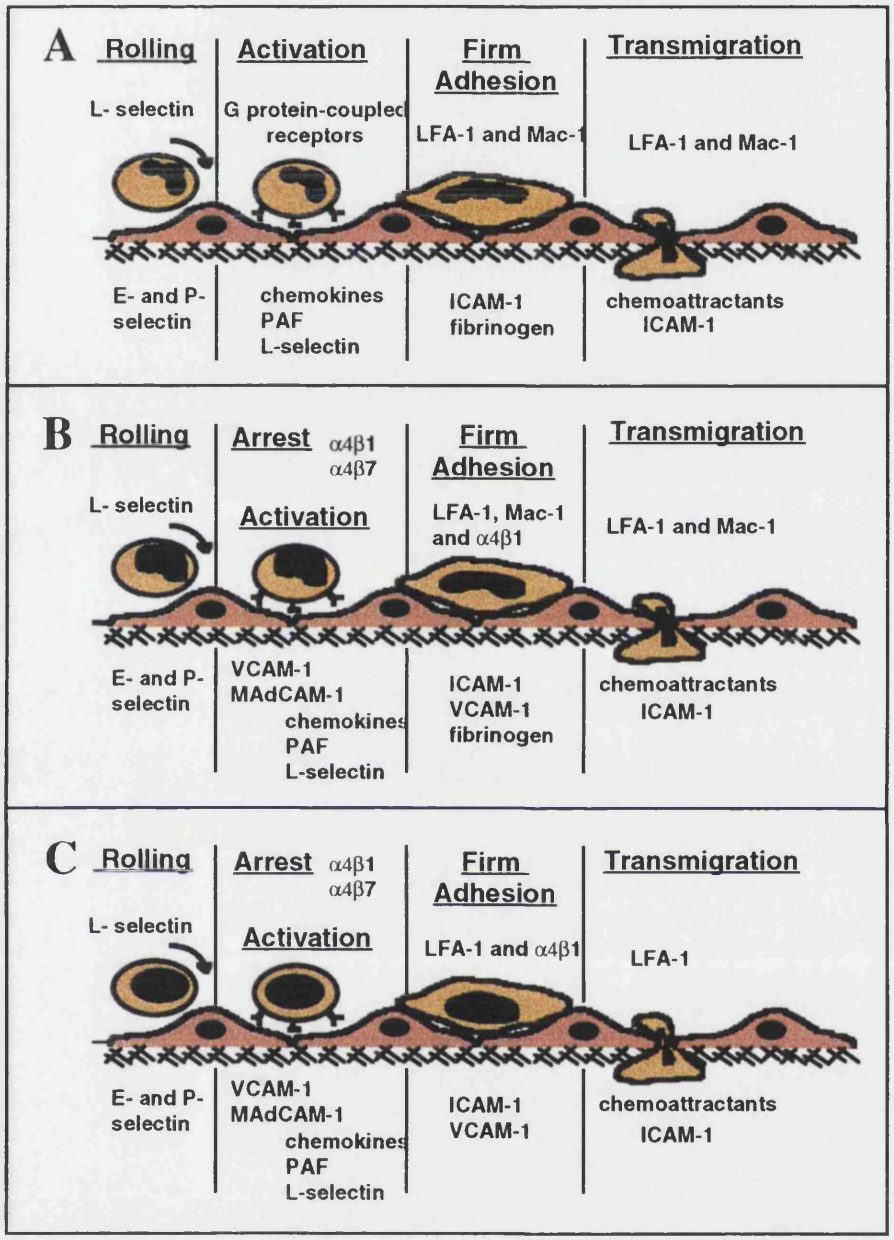

Figure 1.1: Schematic diagram of the leukocyte adhesion cascade. A) Neutrophils, **B)** monocytes and **C)** lymphocytes roll along stimulated endothelium by ligand interactions of their selectins and α_4 integrins (B and C only). Once cells are activated, cells arrest on endothelium and firmly adhere via LFA-1, Mac-1 (β_2 integrins), and $\alpha_4\beta_1$. Cells then migrate using β_2 integrins in response to chemoattractants in the subendothelial tissue.

1.3 THE LEUKOCYTE ADHESION CASCADE

1.3.1 LEUKOCYTE "ROLLING"

Leukocyte "rolling" is a phenomenon described for the reversible interaction of leukocytes, under flow conditions, with recently stimulated vascular endothelium. Rolling is mediated primarily by the interaction of a family of adhesion molecules, called the selectins. The selectin family comprises three members, designated L (leukocyte), E (endothelial) and P (platelet), which represents the cell type in which they were originally discovered (reviewed in (Kansas, 1996)). P-selectin is also expressed on the surface of endothelial cells, and L-selectin is expressed on all circulating leukocytes except for a subpopulation of memory lymphocytes. They are also known as CD62L, CD62E and CD62P, respectively.

L-selectin was originally described as a murine lymphocyte homing receptor, but has subsequently been shown to be expressed on other leukocytes and to participate in the response of leukocytes to inflammation or injury (reviewed in (Kansas, 1996)). L-selectin is constitutively expressed on leukocytes and is proteolytically cleaved upon activation of the leukocytes (Kishimoto et al., 1989) by a unique metalloproteinase (Preece et al., 1996). In contrast to L-selectin, P- and E-selectin are not constitutively expressed but are induced upon cell stimulation. P-selectin is stored in preformed Weibel-Palade bodies in endothelial cells and the α granules of platelets (McEver et al., 1989) and is rapidly (<20 min) mobilised to the plasma membrane to bind leukocytes in response to mediators of acute inflammation, such as thrombin and histamine (Kansas, 1996). The induction of E-selectin on vascular endothelial cells by cytokines such as IL-1, lipopolysaccharide (LPS), TNF is maximal at 3-6 hours and requires *de novo* mRNA and protein synthesis (Bevilacqua et al., 1989). Together, the selectins, by binding to their ligands on opposing cells, mediate tethering of flowing leukocytes to the vessel wall. Once tethered, fast on/off rates (Alon et al., 1997; Puri et al., 1997) of the selectins/ligands interactions cause the leukocytes to "roll" along the vessel wall (von Andrian et al., 1991).

The general extracellular structure of a selectin is an N-terminal domain homologous to C-type (Ca^{2+}-dependent) lectins, followed by an epidermal growth factor (EGF)-like motif, and a variable number of consensus repeats similar in sequence to those in complement-regulatory proteins (reviewed in (McEver, 1994)).The N-terminal lectin domains of selectins are primarily responsible for ligand binding and recognise on their

counter-receptor carbohydrate determinants, which are sialylated, fucosylated and/or sulphated (or all three) (Lasky, 1992; Rosen, 1993). The carbohydrate structures recognised by the selectins are distinct but closely related to sialyl Lewis^x (sLe^x) and its isomer sialyl Lewis^a (sLe^a). In particular, L- and P-selectin carbohydrate ligands are O-linked to mucin-like molecules. Mucins are serine- and threonine-rich proteins that are heavily O-glycosylated. L-selectin recognises two mucins from murine high endothelial venules (HEV), glycosylation-dependent cell adhesion molecule (GlyCAM)-1 (Lasky, 1992; Rosen, 1993) and CD34 (Baumhueter et al., 1993). In addition, L-selectin binds to the addressin, MAdCAM-1 (mucosal addressin cell adhesion molecule-1), on mucosal lymph node HEV (Berg et al., 1993). No ligand for L-selectin has yet been identified on inflamed human vascular endothelium as: human GlyCAM-1 has not been identified and murine GlyCAM-1 appears to be a soluble molecule (Varki, 1997); and although CD34 is a transmembrane glycoprotein expressed on endothelia throughout the vasculature (Baumhueter et al., 1994), it only appears to be correctly glycosylated for L-selectin recognition on HEV of human lymph nodes (Varki, 1997). Therefore, alternative L-selectin ligands, as yet unidentified, must be present on endothelia to mediate "rolling". The most well described P-selectin ligand is the P-selectin glycoprotein ligand (PSGL)-1, which is a disulphide-linked dimer (Moore et al., 1992; Sako et al., 1993). PSGL-1 also binds to E-selectin but with much lower affinity (see (Varki, 1997)). In contrast to the above sialomucin ligands with O-linked glycosylation, the E-selectin ligand-1 (ESL-1) is N-linked glycosylated and shows homology to chicken fibroblast growth factor receptor (Steegmaier et al., 1995). L- and P-selectin can also recognise heparan sulphate glycosaminoglycans (GAGs) which lack sialic acid and fucose (Nelson et al., 1993) and certain sulphated glycolipids, such as sulphatides (Aruffo et al., 1991).

The importance of selectins in leukocyte recruitment and lymphocyte recirculation has been known for a number of years. A study by von Andrian *et al.* (von Andrian et al., 1991), using antibodies to L-selectin was able to show that interaction of neutrophils with endothelium via L-selectin was crucial for neutrophil rolling. Furthermore, this study showed that L-selectin interactions were also crucial for integrin-mediated firm adhesion to endothelium. Thus, it appeared that L-selectin interactions preceded and were essential to integrin interactions (hence the 'leukocyte adhesion cascade') (von Andrian et al., 1991). Now two patients have been identified which are genetically deficient in sLe^x (Etzioni et al., 1992). These Leukocyte Adhesion Deficiency (LAD) II patients lack ligands for E- and P-selectin and show an inability to recruit neutrophils to sites of inflammation. Recently, a more defined role for selectins in leukocyte

adhesion/migration has been described based on studies of selectin-deficient mice. L-selectin-deficient mice have impaired leukocyte rolling and lymphocyte homing *in vivo* (Arbones et al., 1994); and leukocyte rolling and extravasation are also severely compromised in P-selectin-deficient mice (Mayadas et al., 1993). However, when neutrophil recruitment into inflamed peritoneum was analysed in L- and P-selectin-deficient mice at later time points, normal cell numbers were observed. These observations suggested that E-selectin, expressed later, may be able to mediate rolling in the absence of the other selectins. In E-selectin-deficient mice, however, no real defect in leukocyte recruitment was observed (Labow et al., 1994), suggesting that in the presence of other selectins, E-selectin may be redundant in this inflammatory model. Considering together the functional data from these selectin-deficient mice, it appears that P- and L-selectins are initially required for rolling with an involvement of E-selectin at later time points. This data correlates well with the kinetics of selectin expression in inflammation.

In addition to selectin-mediated rolling, lymphocytes and monocytes have also been shown to roll using members of the integrin family of adhesion (see section 1.3.2.1 below). The $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins are able to mediate lymphocyte and monocyte rolling by their interactions with ligands VCAM-1 and MAdCAM-1 ($\alpha_4\beta_7$ only) (Alon et al., 1995; Berlin et al., 1995; Luscinskas et al., 1994). The interaction of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ with ligand has also been shown to cause arrest of these cells on endothelium. Neutrophils express very little α_4 integrins and, even when expressed, these integrins are not able to mediate rolling or arrest of these cells (Reinhardt et al., 1997). Instead, neutrophils appear to rely exclusively on activation of their β_2 integrins for effective firm adhesion to endothelium; whereas monocytes and lymphocytes can use arrest on α_4 as a precedent to activation-dependent firm adhesion.

1.3.2 ACTIVATION-DEPENDENT FIRM ADHESION

The mechanism of leukocyte 'rolling' enables the cells to slow down in the blood flow and also increases the number of contacts the cells make with endothelium, thereby increasing their exposure to potential stimuli. Once stimulated, leukocytes have been observed, under conditions of flow, to arrest completely, firmly attach to and spread on endothelium, prior to transmigration through intercellular junctions. The firm adhesion step is mediated by the integrins.

1.3.2.1 INTEGRINS

The term integrin is used to describe cell surface receptors which could "integrate" the intracellular cytoskeleton with the extracellular matrix. Integrins are expressed on almost all cells and are a large family of glycoproteins which form non-covalently associated α/β heterodimers (Hynes, 1992). To date there are 16 α subunits (~150-210 kDa and 8 β subunits (95-110 kDa; β_4~205 kDa). Out of those subunits, 22 α/β heterodimer combinations have been described (**Figure 1.2**). Of these, 13 are expressed on leukocytes (**Figure 1.2,** red lines), and the β_2 and β_7 integrins are exclusive to leukocytes (reviewed in (Stewart et al., 1995)).

a) Integrin α subunits

The α subunits, α_{IIb}, α_V, and α_3-α_9, share a general structure of seven extracellular repeats (I-VII) of about 60 amino acids each, with four (or three for α_4) putative divalent cation-binding sites (repeats IV-VII). This year the structure of the extracellular region of the integrin α subunit has been remodelled (Springer, 1997). In the new model, these N-terminal ~440 amino acids of the α subunit contain seven sequence repeats which are predicted to fold into a β-propeller domain around a central axis. The integrin β-propeller structure shows most homology to the trimeric G protein β subunit. The four β_2 integrin α subunits: α_L (CD11a; LFA-1), α_M (CD11b; Mac-1; CR3), α_X (CD11c; p150,95; CR4), and α_D (CD11d), together with α_1, α_2, and α_E, contain an additional inserted (or 'I') domain of ~200 amino acids, between repeats II and III. This 'I' domain is homologous to the A domain originally described for von Willebrand factor (vWF) (see (Colombatti and Bonaldo, 1991)). In the β-propeller model, the 'I' domain, which is structurally homologous to the G protein α subunit, is tethered to the top of the β-propeller domain and folds independently to the rest of the α subunit (Huang and Springer, 1997; Springer, 1997).

In 'I' domain-containing α subunits, there are three, instead of four, divalent cation-binding sites (domains V-VII); however, it has since been shown in Mac-1 that the 'I' domain itself contains a novel divalent cation-binding site (Michishita et al., 1993; Zhou et al., 1994). This has been called a MIDAS motif, or Metal Ion Dependent Adhesion Site, and contains a Mg^{2+} coordination site represented by the sequence of amino acid DxSxS. Confirmation of the functional ability of this site to bind cations came from the crystal structures of isolated LFA-1 and Mac-1 'I' domains (Lee et al., 1995; Qu and Leahy, 1995), in which a Mg^{2+} ion was directly coordinated by the DxSxS sequence. As well as being a divalent cation binding site, the 'I' domain also

represents a major ligand binding site in the integrins in which it is found (Diamond et al., 1993; Kamata et al., 1994; Kern et al., 1994; Landis et al., 1993; Landis et al., 1994; Michishita et al., 1993; Randi and Hogg, 1994; Tuckwell et al., 1995; Zhou et al., 1994). In the non-'I' domain containing integrin $\alpha_{IIb}\beta_3$, ligand binding can be mediated by the divalent cation binding sites (D'Souza et al., 1990). In the 'I' domain-containing integrin LFA-1 ($\alpha_L\beta_2$), an additional binding site has been located in the cation binding sites V/VI (Stanley et al., 1994), suggesting that there may be conserved binding sites in the cation-binding sites of all α subunits. In addition, for $\alpha_4\beta_1$, the binding site for VCAM-1 has been identified in regions other than the cation-binding sites (Kamata et al., 1996). The β-propeller model predicts that the ligand binding site for non-'I' domain-containing integrins is on the upper face and the Ca^{2+}-binding sites on the lower surface. This has been confirmed by modelling the $\alpha_4\beta_1$ ligand-binding sites on the β-propeller model (Irie et al., 1997), and suggests that the ligand binding sites may be distinct from the cation binding sites. However, Springer suggests that a putative Mg^{2+} may bind to the upper surface of the β-propeller. Beyond domain VII and the rest of the extracellular portion of the α subunits, is the transmembrane sequence and the short cytoplasmic domains. All α subunit cytoplasmic domains contain a conserved membrane proximal motif, GFFKR (single letter amino acid code), which is thought to participate in modulating integrin activation state, signal transduction and heterodimer formation (O'Toole et al., 1994).

b) Integrin β subunits

The β subunit of integrins have an approximately 700 amino acid extracellular domain, a hydrophobic transmembrane region and a cytoplasmic tail of varying length. Within the extracellular domain, the carboxy-terminal half is a cysteine-rich domain of four consecutive repeats each with internal disulphide bonds. Studies with the β_3 subunit suggest that these cysteine bonds may be important for integrin structure (Calvete et al., 1991). The N-terminal half of the β subunit contains a conserved region, thought to contain an additional MIDAS motif (Lee et al., 1995), which suggests that the β subunit may also contain a ligand binding and cation-binding site (Cierniewski et al., 1994; Lee et al., 1995). Two regions within the cytoplasmic domains of integrin β subunits have been shown to be involved in integrin function. One of these is the highly conserved NPxY motif (O'Toole et al., 1995), found twice in all β subunits, except for β_4. The other region is the conserved membrane-proximal sequence KLLxxxxD, where x represents any amino acid. The role of cytoplasmic sequences in

regulating integrin function will be discussed in section 1.3.3.4 b). The structures of representative integrin α and β subunits are represented in **Figure 1.3**.

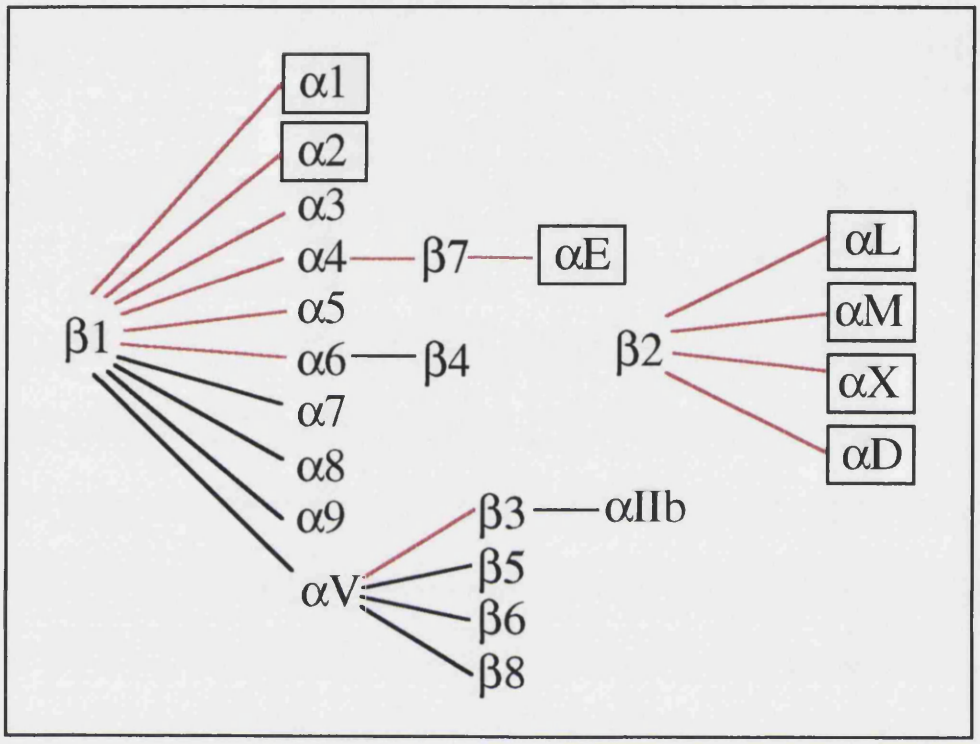

Figure 1.2: The integrin family of adhesion molecules. Lines denote known integrin α and β subunit heterodimers, and red lines indicate those heterodimers expressed on leukocytes. Boxes indicate α subunits which contain 'I' domains.

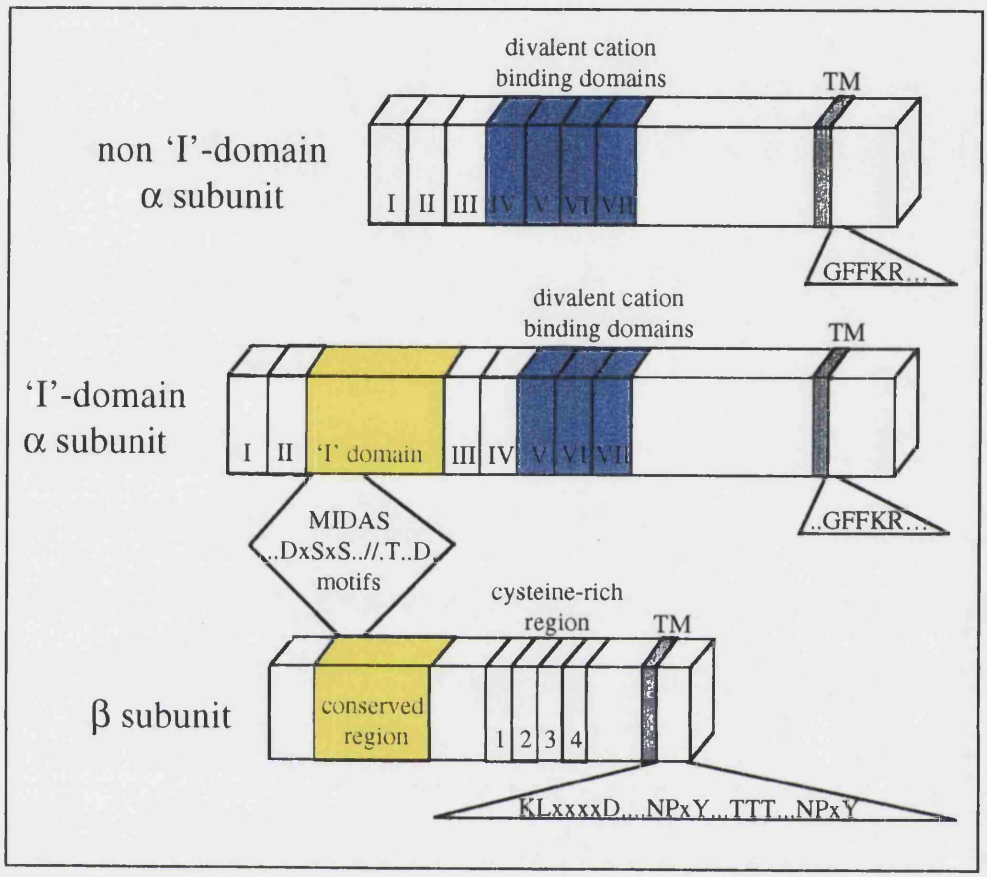

Figure 1.3: Integrin α and β subunit structure. The N-terminal ~440 amino acids of the integrin α subunit is composed of 7 repeats which are predicted to fold into a β-propeller domain (Roman numerals I-VII). The last three (or four), repeats IV (or V)-VII, contain divalent cation binding sites (blue). For those integrin which contain an 'I' domain, inserted between repeats II and III (yellow), there is an additional cation binding site, known as the MIDAS motif. The β subunit contains an homologous region to the MIDAS motif in its N-terminus (yellow). In thew C-terminal half of the β subunit there are cysteine-rich repeats (numbered 1-4). Important motifs in the α and β subunit cytoplasmic tails are represented as single letter amino acid codes as indicated on the figure.

1.3.2.2 Leukocyte Integrin Ligands

a) Immunoglobulin Supergene Family

Integrin ligands on endothelium are predominantly members of the immunoglobulin supergene family (IgSF). The first of which described was Intercellular Adhesion Molecule (ICAM)-1, which interacts with LFA-1 (Marlin and Springer, 1987) and is widely expressed on cells, including myeloid, lymphoid and endothelial cells (reviewed in (Gahmberg et al., 1997)). On endothelial cells ICAM-1 expression is upregulated by various cytokines (reviewed in (Gahmberg et al., 1997)). In addition, two homologous IgSF members, ICAM-2 and ICAM-3 were also found to be ligands for LFA-1 (de Fougerolles and Springer, 1992; de Fougerolles et al., 1991). ICAM-2 is more restricted in its expression than ICAM-1, but it is constitutively expressed by endothelium. ICAM-3 has five Ig-like extracellular domains, similar to ICAM-1, and was constitutively expressed on resting leukocytes (de Fougerolles and Springer, 1992; Fawcett et al., 1992). Recently, two new ICAM family members have been identified. ICAM-4 is unlike ICAMs 1-3 as it is restricted in its expression to erythrocytes and erythroid precursors (Bailly et al., 1995). ICAM-4 binds to CD11/CD18 (β_2) integrins but its function is still not known. ICAM-5, also known as telencephalin, is strongly expressed by the grey matter of the telencephalon (Mizuno et al., 1997; Yoshihara et al., 1994). Like other ICAMs, ICAM-5 is a ligand for LFA-1, although the functional significance of this interaction is not known (Mizuno et al., 1997).

Previous work has identified the first two domains in ICAM-1, ICAM-2 and ICAM-3 as most important in binding to LFA-1 (Gahmberg et al., 1997; Holness et al., 1995; Staunton et al., 1990). In addition, ICAM-1 and a peptide from domain 1 of ICAM-2 have also been shown to bind to Mac-1 (Diamond et al., 1991; Li et al., 1995), although the binding of Mac-1 to ICAM-1 is to a site in domain 3, distinct from the domain 1 LFA-1 binding site. The first domain of ICAM-1 also binds to fibrinogen (Duperray et al., 1997), although the site is also distinct from the LFA-1 binding site. It is envisaged that fibrinogen bound to ICAM-1 may be able to form a bridge between Mac-1 on myeloid cells and endothelium. This binding may, therefore, be an important additional pathway of recruiting leukocytes into inflamed tissues, as fibrinogen binding to endothelium results in increased leukocyte adhesion and initiation of migration (Languino et al., 1995).

Two other IgSF members are also involved in leukocyte adhesion to endothelium:

1) Vascular cell adhesion molecule (VCAM)-1 is an IgSF member whose expression is induced on endothelial cells by cytokines and is a ligand for $\alpha_4\beta_1$ (VLA-4) (Springer,

1994) and $\alpha_4\beta_7$ (Rüegg et al., 1992). It contains 7 (or 6) Ig-like domains and integrin binding sites are found in domains 1 for the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, with a an essential contribution from sequences in domain 2 (Newham et al., 1997). An additional binding site for $\alpha_4\beta_1$ is found in domain 4 of the 7 domain VCAM-1. Six domain VCAM-1 is the product of alternative gene splicing where domain 4 is absent and hence only contains the single binding site in domain 1. Expression of the six domain VCAM-1 is only minor compared to the 7 domain form, suggesting that the predominant VCAM-1 is the 7 domain form.

2) Mucosal addressin cell adhesion molecule (MAdCAM)-1, found on high endothelial venules (HEV) in some lymphoid tissues, is also a member of the IgSF (Briskin et al., 1993). It contains three immunoglobulin-like domains and a mucin-like region between Ig-like domains 2 and 3. MAdCAM-1 binds the integrin $\alpha_4\beta_7$, but not $\alpha_4\beta_1$ (Berlin et al., 1993). This is in addition to the binding of L-selectin, previously mentioned, to the mucin-like domain of MAdCAM-1 (Berg et al., 1993). Models of the IgSF family members involved in leukocyte adhesion to endothelium are shown in **Figure 1.4**.

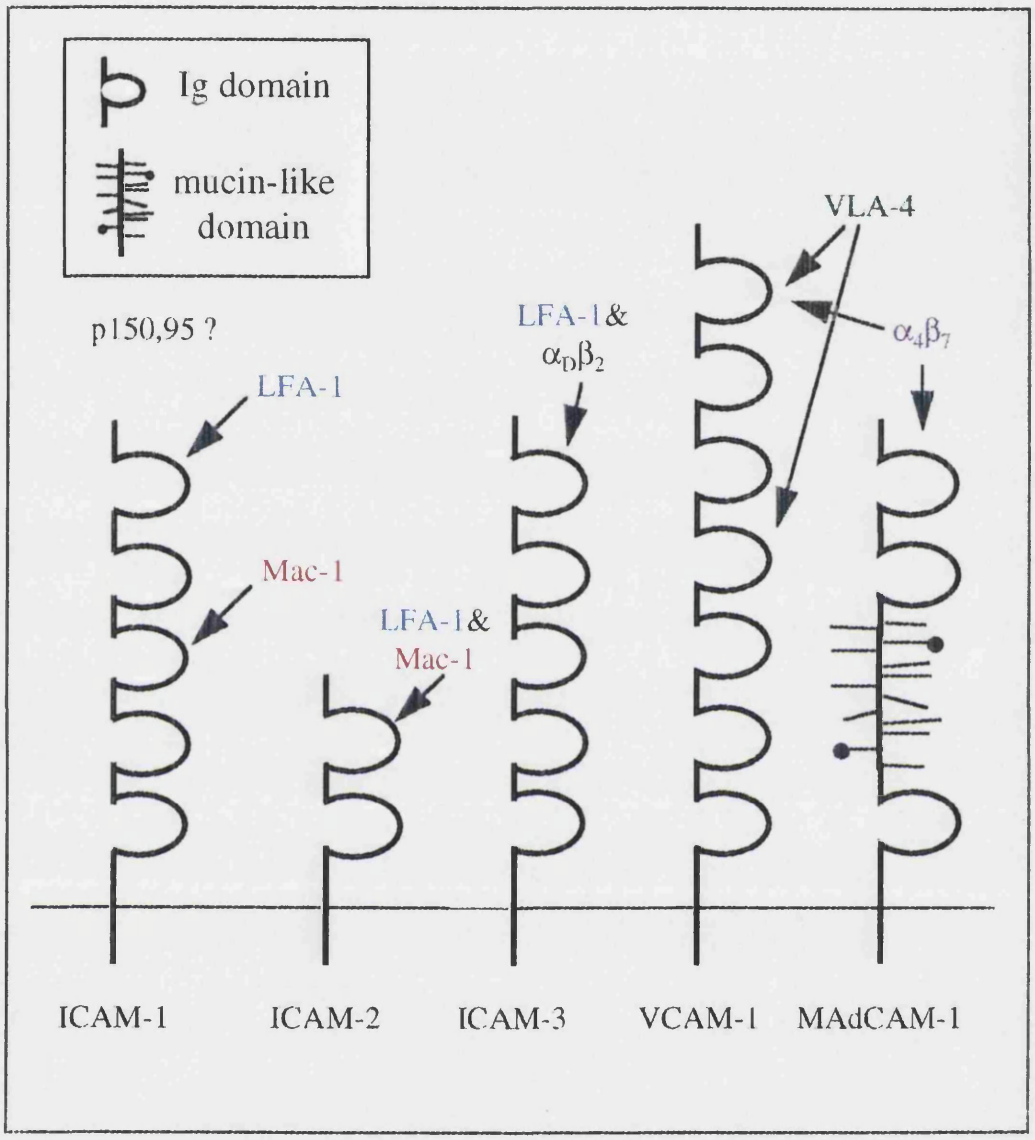

Figure 1.4: Schematic illustration of the immunoglobulin supergene family (IgSF) members found on the surface of the endothelium. Integrins which bind to ICAM-1, -2, -3, VCAM-1 and MAdCAM-1 are indicated on the diagram. The arrows represent the domains in the specific IgSF members to which these integrins bind.

b) Alternative integrin ligands on endothelium

Additional to those of the IgSF, alternate α_4 ligands on endothelium are suggested by the observation that antibodies to α_4 block adhesion to TNFα–stimulated endothelium more efficiently than antibodies to VCAM-1 (Vonderheide and Springer, 1992). The CS-1 fragment of fibronectin has been shown to be an alternate ligand for α_4 on endothelium (Elices et al., 1994), however, antibodies to fibronectin failed to inhibit the binding of leukocytes to TNFα–stimulated endothelium (Vonderheide and Springer, 1992), suggesting yet another α_4 ligand may exist. These experiments, however, have not been thoroughly repeated and may prove the result of artifacts of the experimental systems used.

The lack of complete inhibition of Mac-1 dependent adhesion to endothelium using antibodies to ICAM-1 suggested the possibility that other ligands existed for Mac-1 also (Diamond et al., 1990; Lo et al., 1989). The observations that fibrinogen can bind to both ICAM-1 (see above) and Mac-1(Wright et al., 1988) suggests that fibrinogen may be an alternate Mac-1 ligand. However, another ligand may well exist as, in ICAM-1-deficient mice neutrophils still migrate into inflamed peritoneum (Sligh Jr. et al., 1993). Another alternative Mac-1 ligand is the cleaved third component of complement, iC3b (Arnaout et al., 1983), which binds to Mac-1 when it is immobilised on endothelium *in vitro* and *in vivo* (reviewed in (Carlos and Harlan, 1994)). Other Mac-1 ligands, such as fibrin (Loike et al., 1995), which may be deposited on endothelium, and the coagulation Factor X (Altieri and Edgington, 1988), present in plasma, may also be relevant to leukocyte adhesion to endothelium, although this remains to be tested. In addition to these known ligands, Mac-1 also binds to several other proteins, including high molecular weight kininogen (HMWK) (Wachtfogel et al., 1994), haptoglobin (El Ghmati et al., 1996), elastase (Cai and Wright, 1996), myeloperoxidase (Johansson et al., 1997), and the hookworm protein, NIF (Moyle et al., 1994). Furthermore, Mac-1 is able to bind to LPS (Wright and Jong, 1986), heparin and heparan sulphate (Coombe et al., 1994; Diamond et al., 1995), and oligodeoxynucleotides (Benimetskaya et al., 1997). Most of these Mac-1-binding molecules inhibit the binding of Mac-1 ligands, such as fibrinogen and, suggesting they may play a role in regulating Mac-1-mediated adhesion. For example, the binding of elastase to Mac-1 decreases neutrophil adhesion to fibrinogen and promotes migration (Cai and Wright, 1996), presumably via proteolytic cleavage of the extracellular matrix. In general, these Mac-1 binding molecules may prove to be important regulators of integrin-dependent adhesion, but much remains to be learned about their mode of action.

1.3.3 INTEGRIN ACTIVATION

1.3.3.1 'INSIDE-OUT' ACTIVATION

In contrast to adhesion molecules expressed on endothelium, quantitative changes in integrin are less important than qualitative alterations in function. In fact, an increase in integrin expression is inadequate for inducing adhesion (Diamond and Springer, 1994). Leukocytes (and other cells) constitutively express integrins; however, they freely circulate in the blood without adhering to each other or the vessel wall. This suggests that integrins must be activated in order to be functional.

It has been described for many years that, in *in vitro* T lymphocyte experiments, phorbol esters and cross-linking of relevant cell surface receptors such as the T cell receptor/CD3 complex (Dustin and Springer, 1989) or CD2 (van Kooyk et al., 1989) result in increased LFA-1 adhesion to ICAM-1. Similarly, activation of B lymphocytes through their immunoglobulin receptors (Dang and Rock, 1991), results in LFA-1/ICAM-1-dependent adhesion. Mac-1 on the surface of myeloid cells requires a similar activation in order to adhere to ligand. Activation of neutrophils with chemoattractants, chemotactic peptides, adenosine nucleotides (Freyer et al., 1988), or phorbol esters induces neutrophil adhesion to Mac-1 ligands iC3b (Detmers et al., 1989; Wright and Jong, 1986), fibrinogen (Wright et al., 1988) or ICAM-1 (Diamond et al., 1990; Smith et al., 1989). β_2 integrins are not unique in their requirement for activation in order to function; both β_1 and β_3 integrins are similarly regulated. Platelets are only able to bind ligand after exposure to inflammatory agonists, such as thrombin, adenosine diphosphate (ADP), or platelet activating factor (PAF) (Diamond and Springer, 1994). In addition, cross-linking of cell surface molecules CD2 or CD3 on lymphocytes, or treatment with phorbol esters, increases the adhesiveness of $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins (Chan et al., 1991; Shimizu et al., 1990).

More recently, the interaction of leukocyte L-selectin with ligand, which precedes integrin activation, has been shown to stimulate integrin-mediated adhesion. Cross-linking of neutrophil L-selectin with specific antibodies results in the upregulation of β_2 integrin adhesion (Simon et al., 1995) that can trigger arrest of the cells under flow (Gopalan et al., 1997). These findings have been extended to lymphocytes, where the interaction of L-selectin with either ligand or cross-linking with L-selectin antibodies stimulates β_2 and β_1 integrin adhesion to ICAM-1 and fibronectin, respectively (Giblin et al., 1997; Hwang et al., 1996). These experiments, using models of flow and the *in vivo* L-selectin ligand, GlyCAM-1, suggest that integrin activation by L-selectin cross-linking may be physiologically relevant.

However, all of the above experiments were performed *in vitro* and their validity as modulators of integrin function *in vivo* remains to be determined. One of the criteria for integrin activation *in vivo* would be that the modulating factor must be present at the localised site of inflamed endothelium. An alternative or additional mechanism of integrin activation *in vivo* is suggested by the sensitivity of lymphocyte binding to HEV *in vivo* to pertussis toxin (Bargatze and Butcher, 1993), implicating the seven membrane-spanning receptors of the rhodopsin family in modulating integrin adhesion. The receptors for PAF, fMLP, C5a and the chemoattractant cytokines (chemokines) are of this type and are coupled to heterotrimeric G proteins, through which signalling occurs with rapid kinetics (Murphy, 1994). Thus, the chemoattractants which direct migration of leukocytes from the endothelial surface into the surrounding tissues via a concentration gradient were considered appropriate candidates. As described above, chemoattractants, such as the formylated tripeptide fMLP (e.g. (Smith et al., 1989)), PAF, expressed by activated endothelium, and the complement component C5a (Tonnesen et al., 1989), are able to induce integrin-dependent adhesion *in vitro*.

In recent years, greatest interest has been focused on the chemokines (reviewed in (Baggiolini et al., 1994)). In favour of chemokine activation of integrin *in vivo* is the localised binding on endothelium of the CC chemokines macrophage inflammatory protein-1β (MIP-1β) (Tanaka et al., 1993), and monocyte chemoattractant protein-1 (MCP-1) (Fuentes et al., 1995), and the CxC chemokine IL-8 (Rot, 1992). The association of IL-8 with heparan GAGs has also been shown to enhance its chemotactic activity (Rot, 1992; Webb et al., 1993), suggesting that localised binding of these chemoattractants may be essential for optimal function.

1.3.3.2 CHEMOKINES

The large and expanding families of C, CC and CxC, and the recently identified Cx_3C chemokines (reviewed in (Baggiolini et al., 1994) and (Bazan et al., 1997)) are cell specific and have the potential to recruit leukocyte subpopulations into tissues with greater selectivity than that provided by the other known classical chemoattractants, such as fMLP (Baggiolini et al., 1994; Schall and Bacon, 1994). All chemokines have four conserved cysteines, and the subfamilies can be distinguished according to the position of the first two cysteines, where x represents the amino acid spacing between the first two cysteines. They are all around 10 kDa in molecular weight and the subfamilies are further distinguished by their ability to recruit leukocyte

subpopulations. In general, the CC chemokines are chemotactic for lymphocytes and monocytes; whereas the CxC chemokines are primarily neutrophil chemoattractants.

a) CxC Chemokines

Interleukin (IL)-8 is the prototypic member of this subfamily of chemokines, and was originally isolated from the culture supernatants of stimulated human monocytes. Although, IL-8 is mostly secreted by activated monocytes and neutrophils, it can also by produced by T lymphocytes as well as non-haematopoetic cells such as endothelium and epithelium. Other CxC chemokines can be similarly produced by most of these cell types. IL-8 was identified as a neutrophil agonist based on *in vitro* observations of induction of chemotaxis and the release granular enzymes. The function of IL-8 has since been studied in comparison to the well-characterised neutrophil chemoattractants such as C5a, fMLP and PAF. IL-8, like these chemoattractants, can induce shape change and actin polymerisation, the transient rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$), exocytosis, and the respiratory burst (reviewed in (Baggiolini et al., 1994)). In addition, IL-8 also caused the upregulation of adhesion molecules Mac-1 and p150,95, and the downregulation in L-selectin expression. This upregulation of Mac-1, although not necessary for (Hughes et al., 1992), is accompanied by an increase in the ability of neutrophils to bind ligands on endothelium or to purified fibrinogen and the complement component, iC3b (Carveth et al., 1989; Detmers et al., 1989).

b) CC Chemokines

Monocytes do not generally respond to CxC chemokines, but are highly responsive to CC chemokines. MCP-1, RANTES (regulated on activation normal T cell expressed and secreted), MIP-1α and MIP-1β can all stimulate increases in $[Ca^{2+}]_i$ in monocytes, and MCP-1 also induces the respiratory burst and the increased expression of β_2 integrins, Mac-1 and p150,95 (reviewed in (Baggiolini et al., 1994)). MCP-1 also induces monocyte adhesion to endothelium (Shyy et al., 1993) and can differentially regulate monocyte adhesion to VCAM-1 and fibronectin (Weber et al., 1996), inducing a transient increase in $\alpha_4\beta_1$ adhesive function, followed by sustained $\alpha_5\beta_1$ adhesion. Similarly, the CC chemokines RANTES and MCP-3 differentially regulate β_1 and β_2 integrin adhesion on eosinophils, promoting sustained β_2 integrin adhesion but only transient α_4 integrin adhesion (Weber et al., 1996). How CC chemokines regulate T cell function is not as clear because of conflicting data in the literature. A study by Taub *et al.* (Taub et al., 1993) identified MIP-1α and MIP-1β as T lymphocyte chemoattractants. In particular, MIP-1 α and β are only able to attract T cells activated

through CD3 and not resting T cells. Furthermore, MIP-1α was shown to be more effective on inducing CD8+ (cytotoxic) T cell migration and adhesion to endothelium; whereas MIP-1β was more effective on CD4+ (helper) T cells. RANTES attracted both populations and was more effective at attracting activated T cells, but was only able to induce the adhesion of CD4+ activated T cells. These results, however, are in disagreement with observation by Tanaka *et al.* (Tanaka et al., 1993) that MIP-1β, but not MIP-1α, was chemotactic for resting T cells and enhances adherence of CD8+, but not CD4+, cells to VCAM-1. In terms of integrin-ligand interactions, the CC chemokine, MCP-1, promoted adhesion to fibronectin but not to ICAM-1, VCAM-1 or endothelium (Carr et al., 1996), suggesting that MCP-1 may be required for adhesion through subendothelial matrix subsequent to transmigration. However, another study showed that MCP-1, MIP-1α, MIP-1β, and RANTES were all able to stimulate adhesion of T cells not only to several matrix molecules but also to ICAM-1 and VCAM-1 (Lloyd et al., 1996). These conflicting results may be partly explained by the differing receptor expression and/or usage (Mackay, 1996). In general, the level of expression of chemokine receptors on T cells is 10-100 fold lower than levels on myeloid cells and this may dictate function. Chemokines such as IL-8 and MIP-1α readily induce $\alpha_4\beta_1$-mediated adhesion of lymphocyte cell lines if their receptors are expressed by transfection at high levels of 10^4 to 10^5 /cell (Campbell et al., 1996).

1.3.3.3 Other Cell Surface Receptors Involved in Modulating Integrin Function by Direct Association

It is becoming apparent that integrin function may also be regulated by association of the integrin with other cell surface receptors. An example of this kind of interaction is the functional association of Mac-1 and the GPI-linked urokinase receptor (uPAR, CD87) on monocytic cells (Simon et al., 1996). Both receptors promote degradation of fibrinogen and are adhesive receptors as Mac-1 and uPAR bind fibrin(ogen) and vitronectin, respectively. These two receptors have been shown by resonance energy transfer and co-immunoprecipitation techniques to be physically linked on monocytes (Bohuslav et al., 1995) and neutrophils (Xue et al., 1994). The study by Simon *et al.* (Simon et al., 1996) has provided a functional link between the two receptors, and has identified uPAR as a novel regulator of integrin function. However, the association of uPAR and Mac-1 has been shown to result in a heterogeneous functional response, as occupation of uPAR with vitronectin, promoted Mac-1 degradation of fibrinogen, whereas occupancy of uPAR by exogenous urokinase inhibited Mac-1 function. Whether the heterogeneous functional effect of uPAR is a result of intracellular

signalling or direct physical association with Mac-1 remains to be determined. Despite this, the results suggest that uPAR may be important in regulating Mac-1 adhesion.

Other GPI-linked receptor associated with β_2 integrins are FcγRIIIb and CD14 (reviewed in (Petty and Todd III, 1996)). The association of FcγRIIIb with Mac-1 is essential for efficient IgG-dependent phagocytosis of pathogens, as FcγRIIIb engagement alone is insufficient (Krauss et al., 1994). For CD14, only in the presence of LPS and LPS-binding protein is a physical association with of CD14 with Mac-1 observed and which results in increased adhesion (Petty and Todd III, 1996). FcγRII, although a transmembrane and not a GPI-linked receptor, has also been shown to be functionally associated with Mac-1 (Annenkov et al., 1996).

Another transmembrane cell surface receptor, integrin-associated protein (IAP, CD47), physically associates with $\alpha_V\beta_3$, Mac-1 and $\alpha_{IIb}\beta_3$ (Brown et al., 1990; Lindberg et al., 1996) and has been shown to modulate integrin function. For example, IAP has been shown to promote leukocyte-endothelial interactions mediated by β_2 integrins and CD31 (platelet-endothelial cell adhesion molecule; PECAM-1) (Cooper et al., 1995), although the mechanism of regulation is not understood.

Most recently, CD98 has been shown to be functionally associated with β_1 integrins (Fenczik et al., 1997). CD98 is an early T cell activation antigen and is part of a heterodimer of 120 kDa, composed of an 80 kDa heavy chain, containing the CD98 epitopes, and a 40 kDa light chain. CD98 is physically associated with $\alpha_2\beta_1$ $\alpha_3\beta_1$ $\alpha_5\beta_1$ and $\alpha_6\beta_1$ and when cross-linked on the cell surface results in β_1-dependent adhesion (Fenczik et al., 1997).

1.3.3.4 INTRACELLULAR SIGNALS IN 'INSIDE-OUT' ACTIVATION

The nature of the alterations in integrins occurring when they convert from inactive to active are incompletely understood. A number of monoclonal antibodies have been described that recognise epitopes on activated or ligand-bound integrins (Cabañas and Hogg, 1993; Chen et al., 1994; Diamond and Springer, 1994; Dransfield and Hogg, 1989; Luque et al., 1996; Mould et al., 1995; van Kooyk et al., 1991; Yednock et al., 1995). The existence of these epitopes suggests that conformational changes in integrin result in ligand binding. However, since this has not been definitively proven, the possibility that activation epitopes may arise as a result of ligand binding or integrin clustering still exists.

a) The role of integrin subunit cytoplasmic domains in 'inside-out' activation

The ability of signals emanating from other cell surface receptors to induce expression of integrin activation epitopes and adhesion, suggests that the changes in integrin must occur from the cytoplasmic domain to the extracellular domain. Multiple studies have shown that the cytoplasmic domains of both the α and β subunits have the capacity to influence integrin function. In general, cytoplasmic residues proximal to the transmembrane sequence contain regulatory domains for integrin activation, whereas residues more distal to the membrane appear to transduce signals.

As mentioned in section 1.3.2.1 b), two regions of the β subunit cytoplasmic domains are important in the regulation of integrin affinity. Point mutations in the conserved NPxY (single letter amino acid code, where x is any amino acid) motif in the β_1 and β_3 subunit cytoplasmic domains, distal to the transmembrane, abolished integrin activation (O'Toole et al., 1995). A segment of five amino acids, including three contiguous threonines, found between the two NPxF/Y motifs in the β_2 subunit have been shown to be responsible for sustained adhesion of COS cells to ICAM-1 (O'Toole et al., 1994). However, the same TTT sequence has recently been demonstrated not to be important for LFA-1-mediated chemotaxis in response to the chemokine MCP-1 (Weber et al., 1997) The conserved membrane-proximal sequence (KLLxxxxD) of the β subunit, in contrast, negatively regulates the activation state of integrins, as deletion of this sequence in β_3 results in a constitutive integrin activation (Hughes et al., 1996). A comparable highly conserved membrane-proximal sequence in the α chain (GFFKR) also appears to serve an inhibitory role on integrin activation, as deletion of this sequence in the α_{IIb} and α_L chains also induces constitutive activation (O'Toole et al., 1994; Peter and O'Toole, 1995). Recently, it has been shown that these two membrane-proximal sequences in the α and β subunits are likely to form a salt-bridge (Hughes et al., 1996), stabilising the integrin in a default inactive conformation.

b) Signals generated leading to 'inside-out' integrin activation

In haematopoietic cells, two major pathways for integrin activation arise from signals generated by the engagement of antigen/Fc receptors and G protein-coupled receptors. Common to the mechanism of action of both these types of receptors is activation of a phosphatidylinositol phospholipase, which acts on phosphatidylinositol bisphosphate (PIP_2) to generate diacylglycerol and inositol trisphosphate (IP_3), leading to mobilisation of intracellular calcium $[Ca^{2+}]_i$ and activation of protein kinase C

(reviewed in (Lub et al., 1995) and shown in **Figure 5.12**). Despite this common pathway, the effect of protein kinase C (PKC) inhibitors on integrin-mediated adhesion is variable (reviewed in (Kolanus and Seed, 1997)). T and B cell interaction stimulated through the TCR/CD3 antibody cross-linking is susceptible to inhibition by PKC inhibitors; however, chemoattractant-induced adhesion of neutrophils is not susceptible to PKC inhibition.

An alternative pathway may be generated, as the PIP_2 serves as a substrate for PI 3-kinase, generating PIP_3. Recently, PI 3-kinase has been suggested to mediate integrin activation in leukocytes. Cross-linking of the T cell CD2, CD7, or CD28, each results in activation of PI 3-kinase, together with $\alpha_4\beta_1$ integrin adhesion to fibronectin. In addition, the PI 3-kinase inhibitor wortmannin blocks both β_1 integrin activation and the activation of platelet integrin by thrombin (Shimizu et al., 1995; Zell et al., 1996; Zhang et al., 1996). In neutrophils, chemoattractant-stimulated adhesion is also dependent on PI 3-kinase (Knall et al., 1997).

Small GTP-binding proteins have also been implicated in 'inside-out' integrin activation. R-Ras has been shown to cause cells in suspension to become adherent to the extracellular matrix via integrins (Zhang et al., 1996). The homologous H-Ras has also been shown to inhibit the activation by R-Ras (Hughes et al., 1997), apparently through interference with the mitogen-activated protein (MAP) kinase pathway. The GTPase Rho has also been shown to positively regulate leukocyte adhesion in response to chemoattractants as inhibition of Rho blocked agonist-induced lymphocyte $\alpha_4\beta_1$ adhesion to VCAM-1 and neutrophil adhesion to fibrinogen (Laudanna et al., 1996).

1.3.3.5 'OUTSIDE-IN' ACTIVATION

'Outside-in' activation refers to the action of integrin by direct alterations of the extracellular domain. There are several factors that directly bind to, or are physically associated with, integrin and as such can directly affect integrin function.

a) 'Outside-in' activators

i) Divalent cations

The divalent cation Mn^{2+} has been shown to induce LFA-1 binding to ICAM-1 (Dransfield et al., 1992), Mac-1 binding to fibrinogen (Altieri, 1991), and VLA-4 binding to VCAM-1 (Jakubowski et al., 1995) and fibronectin (Mould et al., 1995). Mg^{2+} has been shown to similarly induce LFA-1 adhesion to ICAM-1 (Dransfield and Hogg, 1989; Stewart et al., 1996) and VLA-2 ($\alpha_2\beta_1$) binding to collagen (Staatz et al.,

1989). Two of the above experiments were performed in cell-free systems (Altieri, 1991; Staatz et al., 1989), suggesting that cation regulation of integrin function occurs in the absence of intracellular signalling. This supports the hypothesis that divalent cations regulate integrin function by direct binding to integrins and causing a conformational change.

For many integrins, Ca^{2+} appears to have an inhibitory role in integrin function. For example, the ability of Mg^{2+} and Mn^{2+} to activate LFA-1 adhesion to ICAM-1 is inhibited by the presence of Ca^{2+} (Dransfield et al., 1992). Ca^{2+} has similar effects on Mg^{2+}-induced adhesion of VLA-2 to collagen (Staatz et al., 1989). Alternately, Ca^{2+} has been shown to play a role in LFA-1 clustering (van Kooyk et al., 1994).

As discussed in section 1.3.2.1, integrins have several potential binding sites: areas around 1) the MIDAS motifs in both the α subunit 'I' domain and the β subunit, and 2) the cation-binding sites in the α subunit (**Figure 1.3**). One reasonable explanation for the inhibitory effects of Ca^{2+} on Mg^{2+} and Mn^{2+} is that they directly compete for the same cation-binding site. Regressional analysis of cation binding to VLA-2 revealed, however, that the binding sites for Ca^{2+} and Mg^{2+} were non-competitive, suggesting that they were binding to distinct sites on the integrin. One explanation for the ability of Ca^{2+} to inhibit integrin function is that the Ca^{2+} binds directly to the ligand binding site. For example, the β_3 integrin, bound Ca^{2+} could be displaced by the binding of an RGD ligand mimetic peptide (D'Souza et al., 1994). Another example showed that in α_5, Ca^{2+} can also compete for binding of the ligand-competent site (Mould et al., 1995). However, in this study, the binding of Ca^{2+} to its high affinity site on α_5 allowed the high affinity binding of Mg^{2+} to a distinct site, suggesting that cation regulation of integrin is complex. The studies with $\alpha_5\beta_1$ support the results found for $\alpha_V\beta_3$, where cations were found to bind to several different sites and subsequently affect ligand binding (Smith et al., 1994). Studies of cation binding requirements of the isolated 'I' domains of Mac-1 and VLA-2 have not provided any further insight into the link between ligand binding and cation binding in integrin, as the results have been contradictory (reviewed in (Humphries, 1996)).

ii) Activating monoclonal antibodies

Activation of β_1, β_2 and β_3 integrins can be mimicked by mAbs that bind to the α or β subunits. The first of such antibodies to be described was the LFA-1 α subunit-specific mAb, NKI-L16, which induced homotypic aggregation of T and B lymphocytic cell lines (Keizer et al., 1988). Three other activating mAbs, KIM127, KIM185 and CBR LFA-1/2, have been described to activate LFA-1 mediated

adhesion, both of which bind to the common β_2 subunit (Andrew et al., 1993; Petruzzelli et al., 1995; Robinson et al., 1992). As they are common to the β_2 subunit they have also been shown to induce binding of Mac-1 to ligand. One LFA-1 α subunit-specific mAb (MEM 83) has the capacity to differentially induce LFA-1 adhesion to ICAM-1 but not ICAM-3 (Landis et al., 1994). This mAb maps to the LFA-1 'I' domain ligand binding site of LFA-1. The fact that KIM127 can induce the binding of isolated Mac-1 to iC3b (Cai and Wright, 1995), suggests that this mAb acts independently of intracellular signals and most likely act by causing a direct conformational change in Mac-1 which results in ligand binding. Similar experiments with anti-β_3 mAbs, have shown that these mAbs can induce fibrinogen binding to fixed or detergent-solubilised α_{IIb} (O'Toole et al., 1990). The anti-β_1 mAb TS2/16 can also induce VLA-5 ($\alpha_5\beta_1$) and VLA-2 ($\alpha_2\beta_1$) to bind to fibronectin and collagen, respectively (Arroyo et al., 1993).

iii) Lipid

Neutrophil Mac-1 can be induced to bind to iC3b ligand by stimulation with an endogenous lipid factor, termed integrin modulating factor (IMF)-1 (Hermanowski-Vosatka et al., 1992). This lipid was the product of stimulated neutrophils and it was proposed, based on the kinetics of IMF-1 synthesis, that upon stimulation with agonists, IMF-1 is released, binds to the neutrophil and stimulates Mac-1 binding. Since then, studies with purified Mac-1 integrin have shown that IMF-1 is able to directly alter the ability of Mac-1 to bind fibrinogen and iC3b-opsonised zymosan (Klugewitz et al., 1997). This lipid was also shown to stimulate LFA-1-mediated homotypic aggregation (Lee et al., 1994). These results support the idea that the lipid is able to directly alter the conformation of Mac-1 (and LFA-1), independent of intracellular cell signalling. A similar lipid factor was shown to stimulate LFA-1 adhesion to endothelium in lymphoid and myeloid cell lines. This factor was termed leukocyte adhesion lipid (LAL) and was found in these cell lines upon stimulation with phorbol esters (Lee et al., 1994); however, it is unclear whether these two factors are identical. Even so, these results suggest that the membrane lipid environment may be important in integrin function.

iv) Ligands

Direct regulation of leukocyte integrin avidity by ligand has been demonstrated for LFA-1 and Mac-1. A peptide from ICAM-2, which specifically binds to purified LFA-1, induces T cell aggregation via LFA-1/ICAM-1 interactions (Li et al., 1993). This

same peptide is also able to bind to Mac-1, but not p150,95, and in doing so stimulates Mac-1/ICAM-1-mediated myeloid cell aggregation (Li et al., 1995). Similarly, the binding of mAbs specific for ICAM-3 is able to stimulate T cell aggregation (Campanero et al., 1993) and binding to endothelium and extracellular matrix proteins, by activation of both β_1 and β_2 integrins (Cid et al., 1994). In addition, occupancy of α_{IIb} (Du et al., 1991; Frelinger et al., 1988) or LFA-1 (Cabañas and Hogg, 1993) integrins by ligand, or ligand mimetics, can induce integrin activation as detected by activation reporter mAbs. This is referred to as the ligand-induced binding site, or LIBS. There are two models for the induction of a LIBS epitope and, therefore, integrin activation by ligand: 1) "Induced fit", where an initial weak receptor/ligand interaction is able to conformationally alter the integrin resulting in a higher affinity state or, 2) integrin, normally fluctuating between low and high affinity states is "stabilised" in a high affinity state by the binding of ligand.

b) Signals generated from leukocyte integrins by 'outside-in' activation

Apart from regulation of integrins by signals generated inside the cell, increasing evidence is emerging that integrins can also transmit signals into the cell. Integrin receptor clustering and integrin occupancy by ligand together activate intracellular signal transduction which result in the activation of a broad array of leukocyte cellular responses, such as chemotaxis, cytokine production, phagocytosis, and gene expression. Signalling molecules are recruited to sites of clustered integrin at points of cell-cell or cell-matrix contacts. In adherent cells, such as fibroblasts and endothelial cells, these sites are called focal contacts or focal adhesions, and contain a number of cytoskeletal proteins, including actin filaments, talin, α-actinin, paxillin, vinculin, and tensin, signalling molecules including tyrosine kinases, PKC, PI 3-kinase, and small GTP-binding proteins, and several adaptor proteins. T lymphocytes express both focal adhesion kinase (FAK) and PYK2 which are phosphorylated in response to activation of β_1 and β_3 integrins (Ma et al., 1997; Maguire et al., 1995) and In leukocytes, integrin ligation can induce an increase in intracellular calcium ($[Ca^{2+}]_i$) and pH, protein phosphorylation, inositol phospholipid turnover, cell spreading, and the induction of gene expression (reviewed in (Rosales and Juliano, 1995)). The activation of FAK tyrosine kinase activity has been shown to activate the mitogen-activated protein kinase (MAPK) signalling pathway. The adaptor proteins Grb2 and Sos link the small GTP-binding protein, Ras, to FAK, which activates another GTP-binding protein, Raf, which in turn activates MAPK via the MAPK kinase, MEK. An additional pathway of MAPK activation occurs through PI 3-kinase. The 85 kDa subunit of PI 3-kinase can

directly associate with FAK, and has recently been shown to activate Raf and the downstream MAPK pathway (King et al., 1997). Activation of MAPK results in increased gene expression and has also been shown to activate PLA_2 (Davis, 1993). Activation of PLA_2 results in the release of arachadonic acid and lysophospholipid which mediate the inflammatory response and are also involved in cytoskeletal rearrangements. Activation of another small GTP-binding protein Rho has been shown to be involved in cytoskeletal reorganisation, seen upon integrin ligation. However, the role of Rho in leukocyte integrin signalling is largely unresolved, as inhibition of Rho in neutrophils has no effect on either actin polymerisation or cell morphology (Ehrengruber et al., 1995).

In addition to the activation FAK and Rho, integrin engagement results in the tyrosine phosphorylation of many proteins and the Src family tyrosine kinases appear to play a key role in this phosphorylation. Ligation of integrins on neutrophils induces the activation of two Src family members, Fgr and Lyn, as well as their translocation to the cytoskeleton (Berton et al., 1994). Activation and translocation of src family kinases appears to be necessary for adhesion, spreading, migration and adhesion-dependent respiratory burst (Lowell et al., 1996). A summary of integrin-mediated signal transduction in outlined in **Figure 1.5**. Many of the pathways activated by integrin ligation and clustering are also activated by 'inside-out' signalling through other receptors (see section 1.3.3.4 a) and **Figure 5.12**), suggesting that successful integrin function may require convergent signals from integrins and other cell surface receptors.

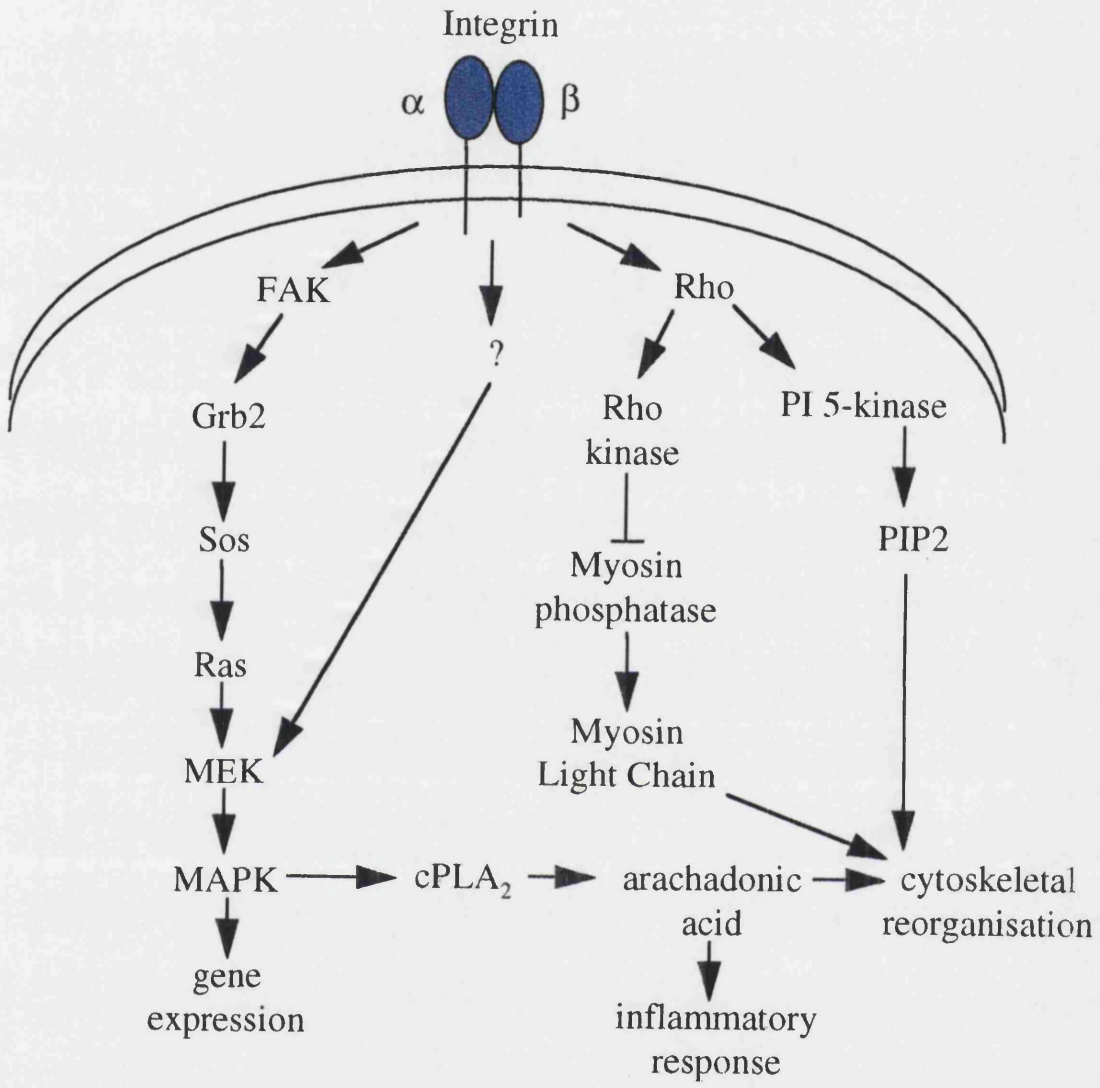

Figure 1.5: 'Outside in' integrin signal tranduction pathways. Selected pathways of signal transduction from integrins are displayed, which result in the activation of gene expression, cytoskeletal reorganisation and the induction of the inflammatory response.

1.3.3.6 INTEGRIN CYTOPLASMIC DOMAIN BINDING PROTEINS INVOLVED IN THE REGULATION OF INTEGRIN FUNCTION

There is a growing list of proteins which are known to associate with integrin cytoplasmic tails and modify overall receptor function.

a) Calreticulin

The calcium-binding protein calreticulin binds to the membrane-proximal highly conserved GFFKR motif present within the α subunit cytoplasmic tail (Rojiani et al., 1991). Mice deficient in calreticulin show defective 'outside-in' signalling, with compromised calcium mobilisation from intracellular stores following integrin engagement (Coppolino et al., 1997). Calreticulin has also been implicated indirectly in 'inside-out' signalling, as anti-calreticulin antibodies have been shown to inhibit the ability of $\alpha_2\beta_1$ to be activated by phorbol esters or by anti-integrin antibodies (Coppolino et al., 1995).

b) Integrin-Linked Kinase

Integrin-linked kinase (ILK) is a serine/threonine kinase that is able to associate with β_1, β_2, and β_3 integrins (Dedhar and Hannigan, 1997; Hannigan et al., 1996). ILK may, like calreticulin be involved in both inside-out and outside-in signalling of integrins. In rat epithelial cells, overexpression of ILK results in decreased adhesion to extracellular matrix, and cell attachment and spreading on fibronectin results in altered ILK activity and anchorage-independent growth (Hannigan et al., 1996). The results for inside-out signalling suggest that ILK may be a negative regulator of integrin function.

c) Cytohesin-1

Cytohesin-1 is a 47 kDa cytoplasmic protein that was found to associate with the intracellular domain of β_2 integrin, using a yeast two-hybrid system (Kolanus et al., 1996). Overexpression of the carboxy-terminal domain of cytohesin-1 inhibits T cell receptor-stimulated adhesion, suggesting that cytohesin-1 may play a regulatory role in β_2 integrin adhesion.

d) β_3 endonexin

β_3 endonexin interacts specifically with the cytoplasmic domain of β_3 integrins (Shattil et al., 1995). Although the physiological role of β_3 endonexin is unknown, it has been

suggested that the binding of endonexin may be able to promote β_3 integrin activation (Kashiwagi et al., 1997)

1.3.3.7 INTEGRIN ACTIVATION - AFFINITY VERSUS AVIDITY

There is evidence that increased leukocyte integrin function, either from 'outside-in' or 'inside-out' activation, may result from two distinct mechanisms: the individual integrin receptors can undergo conformational change, giving rise to higher affinity ligand binding (reviewed in (Stewart and Hogg, 1996)). Higher affinity integrins can be detected by the expression of activation reporter epitopes, such as mAb 24 (Dransfield and Hogg, 1989), or by the binding of soluble ligand. A second mechanism of adhesion is through post-receptor events, such as cell spreading (Faull et al., 1994; Stewart et al., 1996) or integrin clustering on the membrane, thereby increasing the avidity of cell interactions (Stewart et al., 1997). It is probable that both avidity and affinity changes operate together to increase the strength of adhesion.

a) affinity changes in integrin

Most affinity changes in integrin are mediated by 'outside-in' integrin activation. Monoclonal antibodies (mAbs) have been described for integrins which are able to directly activate soluble ligand binding. For example, activating antibodies have been shown to stimulate soluble fibrinogen binding to $\alpha_{IIb}\beta_3$ (O'Toole et al., 1990). Two β_1 mAbs, TS2/16 and 8A2, can also activate binding of soluble ligand binding to $\alpha_2\beta_1$ (Arroyo et al., 1993), $\alpha_4\beta_1$ (Jakubowski et al., 1995) and $\alpha_5\beta_1$ (Faull et al., 1993). Similarly, β_2 activating mAbs can induce the binding of soluble iC3b to purified Mac-1 (Cai and Wright, 1995).

Affinity changes have also been observed in integrins treated with divalent cations, such as Mg^{2+} or Mn^{2+}. Mn^{2+} induces $\alpha_4\beta_1$, (Jakubowski et al., 1995; Jakubowski et al., 1995) to bind soluble VCAM-1. Similarly, the Mac-1 on monocytes can be stimulated to bind soluble fibrinogen and Factor X by treatment with Mn^{2+} (Altieri, 1991) Treatment of T cells with Mg^{2+} ions in combination with chelation of Ca^{2+} stimulates the binding of soluble ICAM-1 to LFA-1 (Stewart et al., 1996). As previously mentioned, the binding of ligand also appears to be able to generate a high affinity state of integrins, such that integrins are positive for expression of 'activation reporter' epitopes recognised by mAbs (Cabañas and Hogg, 1993; Du et al., 1991; Frelinger et al., 1988).

There are fewer examples of activation of high affinity integrin by 'inside-out' signalling. So far, only the stimulation of platelets, with thrombin and ADP, and

myeloid cells, with ADP and the chemoattractant fMLP, allow binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ and Mac-1, respectively (Altieri and Edgington, 1988; Faull and Ginsberg, 1995). Very little is known about the regulation of integrin activation by chemokines, however, treatment of T cells with the CC chemokine MIP-1α failed to induce the binding of soluble VCAM-1 to $\alpha_4\beta_1$ (Jakubowski et al., 1995), suggesting that the promotion of integrin-dependent adhesion by chemokines may occur through changes in avidity.

b) avidity changes in integrin

Avidity changes in integrin permit cell adhesion without the necessity for high affinity receptors. Evidence suggests that these avidity changes occur through integrin receptor clustering and/or post-receptor events such as cell spreading. Stimulation of cells with PKC activators, phorbol esters, has been used extensively to induce cell adhesion. In this model, T cells are induced to spread on fibronectin and ICAM-1 using their $\alpha_5\beta_1$ (Faull et al., 1994) and LFA-1 (Stewart et al., 1996), respectively. Spreading has been observed *in vivo* following T cell interactions with antigen-presenting cells (Donnadieu et al., 1994) and with monocytes and neutrophils transmigrating across endothelium (Beekhuizen et al., 1992), suggesting that the phorbol ester model may have physiological relevance. Phorbol esters have also been able to induce both LFA-1 (Haverstick et al., 1992) and Mac-1 (Detmers et al., 1987) integrin clustering in the absence of ligand binding. Results from studies of signal transduction mediated by integrins would suggest that this clustering important for signalling as clustering is associated with the recruitment of cytoskeletal proteins and signalling molecules to the membrane.

TCR/CD3 cross-linking, similar to the phorbol ester model, induces adhesion without the activation of high affinity integrin status for either $\alpha_4\beta_1$ (Jakubowski et al., 1995) or LFA-1 (Stewart et al., 1996). It is interesting to speculate that $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, and Mac-1, all of which can exhibit soluble ligand binding after 'inside-out' signalling, are regulated by affinity changes and that 'inside-out' activation of other integrins, such as LFA-1 and $\alpha_4\beta_1$, is regulated by changes in avidity. Irrespective of whether activation is mediated by affinity or avidity or a combination of both, it can be assumed that in order for leukocytes to transmigrate through endothelium, some alterations must occur in both the activation state of integrins and their morphology, as migration requires the making and breaking of adhesive interactions and alterations to the cytoskeleton.

1.3.4 LEUKOCYTE TRANSMIGRATION

Many of the leukocytes which have been activated to firmly adhere to endothelium have been observed to crawl over the luminal surface until they encounter the intercellular junction through which they transmigrate. Firm adhesion to endothelium is mediated by a subset of leukocyte integrins. These are the α_4 integrins (not neutrophils) and the β_2 integrins, LFA-1 and Mac-1, although the majority of migration appears to be mediated by the β_2 integrins (Carlos and Harlan, 1994). Patients genetically deficient in β_2 integrins provided early evidence that these integrins were required for leukocyte extravasation (Anderson and Springer, 1987). Neutrophils from these leukocyte adhesion deficiency (LAD) I patients failed to extravasate from blood to inflammatory sites; however, lymphocytes and monocytes from these patients were able to extravasate, presumably by use of their α_4 integrins.

Mice deficient in individual α_M (Mac-1) or α_L (LFA-1) subunits have relatively normal neutrophil responses in which the alternate β_2 integrin takes over the role of the deleted one (Lu et al., 1997; Schmits et al., 1996). In LFA-1-deficient mice, however, there is reduced lymphocyte homing and extravasation, confirming the importance of LFA-1 on these cells. This is also confirmed by recent reports which show that LFA-1 dominates over VLA-4 in firm adhesion to ligand (Porter and Hogg, 1997; van Kooyk et al., 1993). Whether LFA-1 predominates over Mac-1 or vice versa is uncertain, however, one report suggests that LFA-1 is initially involved in unstimulated neutrophil binding to stimulated endothelium, whereas Mac-1 adhesion predominates once neutrophils are stimulated (Smith et al., 1989).

Transmigration between two endothelial cells (or diapedesis), in addition to requiring β_2 integrins, involves the homotypic interaction of CD31 (PECAM-1) on endothelial cells with that on leukocytes. Antibodies to PECAM-1 inhibit the *in vivo* accumulation of rat neutrophils to sites of inflammation (Vaporciyan et al., 1993); however, CD31 is only expressed on a subset of T lymphocytes and may not be involved in T cell extravasation. Antibody blocking of another accessory molecule, integrin-associated protein (IAP, CD47), also results in the inhibition of neutrophil transendothelial migration (Cooper et al., 1995). IAP has previously been discussed as a stimulatory molecule for β_3 and Mac-1 integrins (see section 1.3.3.3).

Once leukocytes have crossed the endothelial layer, they are exposed to the extracellular matrix (ECM). Migration through the ECM to sites of inflammation requires coordinated ligation of β_1 integrins. Perhaps the most important is the

interaction of $\alpha_5\beta_1$ with fibronectin as antibody inhibition of α_5 integrins partially inhibits *in vitro* transendothelial migration (Roth et al., 1995).

Leukocyte extravasation follows a distinct temporal pattern. Migration of neutrophils to sites of inflammation peaks at about 4 hours post-infection, with monocyte accumulation predominating at 12-24 hours. By 48 hours the infiltrating leukocytes are essentially all mononuclear cells (monocytes and lymphocytes) (reviewed in (Carlos and Harlan, 1994)). In addition to the temporal pattern of leukocyte recruitment, there is also selective recruitment of leukocyte subpopulations. One possible mechanism of such selectivity is the expression of specific combinations of adhesion molecules on leukocytes and endothelium. A more favoured explanation is that the selectivity relies on the functions of the multiple stimulating molecules found associated with inflammation. Cytokines and proinflammatory molecules, such as IL-1, TNFα and thrombin, can determine the expression of selectin and IgSF adhesion molecule expression, but chemoattractants are all able to attract distinct patterns of leukocytes to sites of inflammation and complete the steps toward regulated leukocyte extravasation.

1.4 A NEW FAMILY OF CHEMOATTRACTANTS

In recent years, three members of the S100 family of Ca^{2+}-binding proteins have been described as chemoattractants, and like the chemokines they are cell-specific in their function. The murine protein CP-10 (chemotactic protein, 10 kDa; murine S100A8), the first of the S100 proteins to be ascribed such function, is chemotactic for murine neutrophils and monocytes, and human neutrophils (Lackmann et al., 1992; Lackmann et al., 1993). S100L (or S100A2), isolated from bovine lung, is chemotactic for eosinophils, and not neutrophils or monocytes (Komada et al., 1996). Psoriasin (or S100A7), highly upregulated in psoriatic keratinocytes, is chemotactic for CD4+ T lymphocytes and neutrophils, but not for monocytes (Jinquan et al., 1996). All three chemotactic S100 proteins are functional in nanomolar (S100L) or picomolar ranges, identifying them among the most potent chemoattractants described to date. There are two further similarities between these chemotactic S100 proteins and the chemokines. Firstly, both families of proteins are similar in size (~10 kDa); and, secondly, two of the three S100 chemoattractants (CP-10 and S100L) bind to pertussis toxin-sensitive G protein-coupled receptors, as do the chemokines (see (Baggiolini et al., 1994)).

1.4.1 THE S100 FAMILY OF CALCIUM-BINDING PROTEINS

1.4.1.1 S100 PROTEIN STRUCTURE

In addition to the three chemotactic family members mentioned above, another 15 proteins have so far been described as S100 proteins, based on sequence homology. This family are characterised as a group of low molecular weight (10-12 kDa) calcium-binding proteins. Originally, a protein fraction highly abundant in nervous tissue was designated to contain 'S100' proteins as the constituents were soluble in 100% ammonium sulphate (reviewed in (Zimmer et al., 1995)). Subsequent studies revealed that this S100 protein fraction contained predominantly two dimeric proteins, a heterodimer of S100α/S100β (S100a) and a homodimer of S100β/β (S100b) (Isobe et al., 1981). Structurally, S100α and S100β monomers contained two high affinity calcium-binding domains called EF-hands, after the E- and F-helices of parvalbumin. The prototype EF-hand is a loop of 12 amino acids which are spaced to co-ordinate a calcium ion (reviewed in (Schäfer and Heizmann, 1996)). S100 proteins contain a variation on the prototype parvalbumin EF-hand as their N-terminal Ca^{2+}-binding loop is 14 amino acids. On either side of the Ca^{2+}-binding loops are hydrophobic regions and the two Ca^{2+}-binding loops are separated by a central hinge region. The structures of representative S100 family members, S100A8 (MRP-8) and S100A9 (MRP-14), are illustrated in **Figure 1.6**.

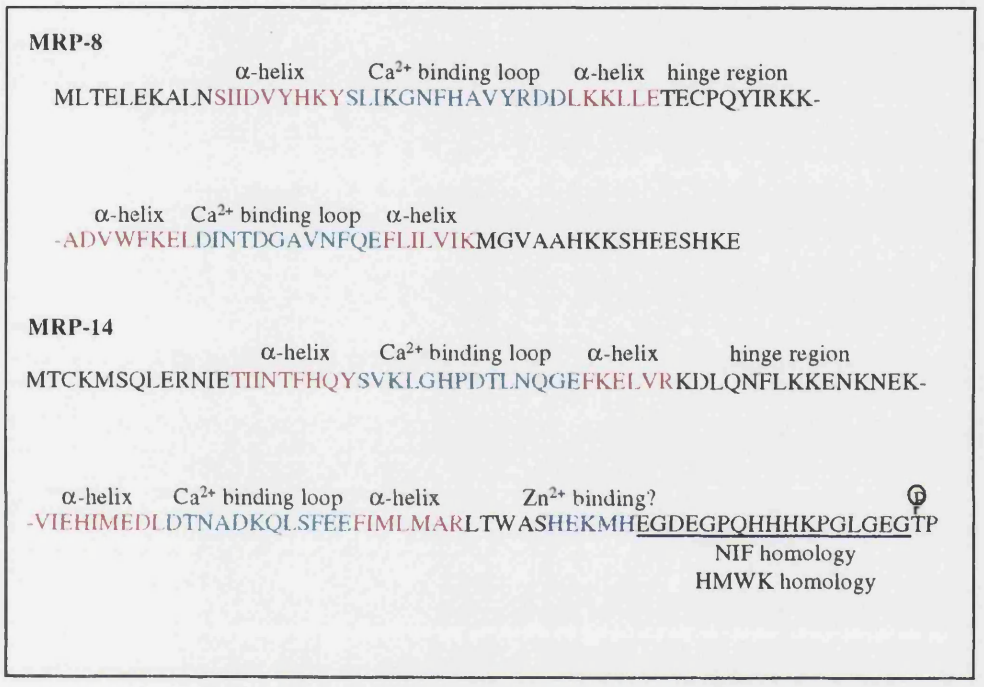

Figure 1.6: Schematic representation of S100 Ca^{2+}-binding proteins, MRP-8 and MRP-14. The amino acid sequences are displayed for human MRP-8 and MRP-14. Calcium-binding EF-hand loops are shown in green. α-helices flanking these loops are indicated in red. The putative zinc-binding site in MRP-14 is indicated in blue, and the regions of homology within MRP-14 to NIF and HMWK are underlined. The MRP-14 phosphorylation site is also indicated on the penultimate amino acid.

1.4.1.2 Chromosomal Localisation

The gene for S100α and fourteen other S100 proteins are localised on human chromosome 1q21. This discovery has led to the introduction of a new nomenclature for the family. The S100a protein has now been designated S100A1 and the other members found on chromosome 1 as S100A2-A13 (Schäfer and Heizmann, 1996; Wicki et al., 1996; Wicki et al., 1996). Two other more unusual members of the family, profilaggrin and trichohyalin are also found on chromosome 1, but their identity as true S100 proteins is still uncertain (Schäfer and Heizmann, 1996). S100β (now S100B) is found on chromosome 21 and the chromosomal locations of the other members, S100P and calbindin 3, have not been identified.

The clustering of the genes for this family of proteins on chromosome 1q21 follows the pattern seen for chemokines, with the genes for CxC and CC chemokines clustered on human chromosomes 4q12-21 and 17q11-12, respectively (cited in (Rossi et al., 1997)). This suggests that members within the S100 protein family whose genes are clustered, like the chemokines, may have functions in common, such as chemotaxis; however, very little information is available regarding the functions of the other S100 protein family members. Only 6 of the members have been identified existing in the extracellular environment, including S100B, S100A4, S100A9, and the three chemoattractants S100A7 (psoriasin), S100A8, and S100A2 (S100L) (reviewed in (Schäfer and Heizmann, 1996)). No chemotactic function has yet been described for S100B, S100A4 or S100A9, and in fact no extracellular function has been described for S100A4. The following introduction on S100 proteins will focus mainly on the two family members, S100A8 and S100A9, and they will be referred to as MRP-8 and MRP-14, respectively. The abbreviation MRP refers to their co-purification with macrophage inhibitory factor (MIF); hence the name MIF-related protein, or MRP. The suffixes -8 and -14 reflect their apparent molecular weights on SDS-PAGE.

1.4.2 Expression of S100 Proteins

S100 proteins are predominantly cytoplasmic in their intracellular expression, although some members are also expressed in the nuclei of cells (reviewed in (Schäfer and Heizmann, 1996)). There are a diverse range of cells which express specific S100 proteins, including nervous tissue, epithelial cells, fibroblasts, muscle, and myeloid leukocytes. The expression of MRP-8 and MRP-14 is restricted to cells of the myeloid lineage (monocytes, neutrophils and some macrophages), certain squamous epithelial cells, keratinocytes and tracheal gland cells. In neutrophils, MRP-8/14 complex comprises ~40% of the total cytosolic protein and in monocytes ~1% (Edgeworth et

al., 1991). Resident tissue macrophages do not normally express MRP-8/14, suggesting that differentiation of monocytes to macrophages is associated with a loss of expression (Hogg et al., 1989; Zwadlo et al., 1988). Furthermore, the immunohistochemical studies in which MRP-8/14 is found associated with the vascular endothelium, the majority of the monocytes migrated into the tissue have lost expression of MRP-8/14 (Hessian et al., 1993; Hogg et al., 1989). As well, cultured monocytes also down-regulate MRP-8/14 (Zwadlo et al., 1988). Under conditions of chronic inflammation, however, macrophages can be induced to express MRP-8/14. For example, MRP-8/14 is expressed in macrophages from patients with rheumatoid arthritis, sarcoidosis, tuberculosis, and onchocerciasis (Edgeworth et al., 1993; Odink et al., 1987; Palmer et al., 1987; Zwadlo et al., 1988). MRP-14 has also been shown to be differentially expressed by macrophages in acutely inflamed tissues (Delabie et al., 1990; Zwadlo et al., 1988). Differential expression of MRP-14 has not similarly been demonstrated in mice; however, LPS-stimulated murine macrophages are induced to differentially express MRP-8 and not MRP-14 (Hu et al., 1996). These results suggest that with the appropriate inflammatory stimuli either the individual proteins or the protein complex may be expressed.

Expression of MRP-8/14 by human epithelial cells is regulated similarly to macrophages. Normal skin is essentially negative except for areas of the pilosebaceous gland (Gabrielsen et al., 1986). In contrast, various mucosal squamous epithelia are constitutively positive (Brandtzaeg et al., 1987; Wilkinson et al., 1988). MRP-8/14 can be induced to be expressed in inflamed skin, such as that found in patients with systemic lupus erthematosus or psoriasis, as well as in cutaneous malignancies (Brandtzaeg et al., 1987; Gabrielsen et al., 1986).

1.4.3 Intracellular Functions of S100 Proteins

Most S100 proteins appear to play an intracellular role during calcium-dependent signalling (Kligman and Hilt, 1988; Schäfer and Heizmann, 1996). They are able to interfere with cell cycle progression, inhibit phosphorylation reactions, or modulate membrane/cytoskeleton interactions in a Ca^{2+}-dependent manner. For example, S100A1 and S100B can regulate the phosphorylation mediated by PKC and can thereby modify the intracellular signal transduction (Baudier et al., 1992). Another S100 protein, S100A10, can inhibit the activity of PLA_2 (and therefore the production of arachadonic acid) by directly binding to it, further supporting a role for S100 proteins in signal transduction (Wu et al., 1997). S100 proteins can also regulate the energy metabolism of cells by modulating target enzyme activities, such as adenylate

cyclase (S100A1), glyceraldehyde-3-phosphate dehydrogenase (S100A6), and fructose-1,6-bisphosphate aldolase (S100A1 and S100B) (Schäfer and Heizmann, 1996). MRP-8/14 has also been shown to inhibit casein kinase II (CKII) and CKI (Murao et al., 1989). CKII has been implicated in the phosphorylation of substrates necessary for normal cellular transcription and translation. The expression of MRP-8/14 correlates well with the differentiation of myeloid cells and a suggestion would be that inhibition of CKII by MRP-8/14 may control normal cell differentiation. These results have failed to be reproduced in our lab using purified MRP-8/14 (Paul Hessian, unpublished results), suggesting that in trying to isolate the true inhibitor of CK, MRP-8/14 may have been inadvertently isolated due to its high abundance in the neutrophil.

Cell shape may also be regulated by S100 interactions, as members of the family have been shown to interact with microtubule proteins and intermediate filaments and influence their polymerisation state (Zimmer et al., 1995). For example, S100A1 and S100B both bind to tubulin and inhibit microtubule assembly (Donato, 1988). MRP-8 and MRP-14 bind to the intermediate filament type III protein, vimentin (Roth et al., 1993). This data suggests that MRP-8 and MRP-14, and other S100 proteins, play a role in modulating the cytoskeleton. Several members of the S100 protein family, including S100A6, S100A10, and S100A11, interact with specific annexins, which in turn are the targets of several kinases and these interactions are also thought to play an important role in intracellular signal transduction (Schäfer and Heizmann, 1996). It is also possible that MRP-8 and MRP-14 may play a role in the inflammatory response of myeloid cells as MRP-8/14 complex, but not individual subunit proteins, are able to bind unsaturated fatty acids, such as arachadonic acid in a Ca^{2+}-dependent manner (Siegenthaler et al., 1997). Alternatively, unsaturated fatty acid binding may be a means by which MRP-8 and MRP-14 are able to associate with the cell membrane.

1.4.4 SECRETION OF MRP-8 AND MRP-14

As mentioned above, MRP-8/14 is primarily a cytosolic protein (Dale et al., 1983; Edgeworth et al., 1991) and, as such, is not normally detected on the cell surface. One monoclonal antibody, 27E10, detects cell surface expressed MRP-8/14 in a subset of monocytes/macrophages in acute, but not chronic, inflammation (Bhardwaj et al., 1992). This antibody recognises only the non-covalently associated heterodimer, suggesting that it is the complex form of MRP-8/14 that is expressed on the cell membrane (Bhardwaj et al., 1992). The cell surface expression of MRP-8/14 is not yet understood as neither MRP-8 or MRP-14 contain a transmembrane sequence.

Recently, several reports have addressed the issue of translocation of MRP-8/14 from the cytosol to the membrane and the cytoskeleton. Conclusions from studies with monocytes are that MRP-8/14 is translocated to the membrane and to the intermediate filament, vimentin, in a Ca^{2+}-dependent manner (Roth et al., 1993) which also depends on the phosphorylation of MRP-14 (van den Bos et al., 1996). Similar Ca^{2+}-and phosphorylation-dependence has been demonstrated for translocation of MRP-8/14 in neutrophils (Guignard et al., 1996; Lemarchand et al., 1992). Phorbol ester stimulation also results in the phosphorylation of MRP-8 and MRP-14 in neutrophils, but this does not result in translocation of the proteins to the membrane (Guignard et al., 1996), suggesting that PKC is not involved in translocation. In fact inhibition of PKC enhanced the amount of translocation observed (Guignard et al., 1996). In addition to not having a transmembrane sequence, neither MRP-8 and MRP-14 (nor any other S100 protein) contain a signal sequence required for secretion via the classical endoplasmic reticulum/golgi route. In this sense, they resemble cytokines such as interleukin-1β (IL-1β) and basic fibroblast growth factor, which are released via an alternative pathway of secretion (Muesch et al., 1990). Therefore, it was suggested that MRP-8/14 may be secreted via this alternative pathway. However, a recent report which revealed active secretion of MRP-8/14 complex, at least from activated monocytes, is distinct from that of IL-1β and is dependent on activation of PKC and an intact microtubule network (Rammes et al., 1997). The observations that the proteins are translocated from the cytosol to the membrane intermediate filaments suggested that this step may be involved in secretion, however, MRP-8/14 secretion was shown to occur independently of translocation (Rammes et al., 1997). This may reflect different functions for intracellular and extracellular MRP-8/14. Furthermore, MRP-14, in addition to the MRP-8/14 complex, has been shown to be secreted from monocytes stimulated with pokeweed mitogen (Lügering et al., 1997). This secretion was susceptible to inhibition by preincubation with a combination of IL-4 and IL-10 cytokines, suggesting an active secretion mechanism.

1.4.5 EXTRACELLULAR FUNCTIONS FOR THE S100 PROTEIN FAMILY

1.4.5.1 MRP-8 AND MRP-14 IN THE EXTRACELLULAR ENVIRONMENT

Several reports have identified MRP-8 (S100A8) and MRP-14 (S100A9) as a complex (MRP-8/14) in the extracellular environment, secreted from stimulated myeloid cells (Burmeister et al., 1986; Murao et al., 1990; Rammes et al., 1997). In addition, MRP-8/14 is found in the serum of patients with cystic fibrosis and other chronic inflammatory conditions, such as rheumatoid arthritis and sarcoidosis (Brüggen et al.,

1988; Bullock et al., 1982). Interestingly, the levels of MRP-14 in cystic fibrosis and rheumatoid arthritis patients are greater than those of MRP-8 (Brüggen et al., 1988), suggesting either MRP-14 is secreted alone as well as in complex, or that it remains soluble after complex dissociation. Originally, the MRP-8/14 complex was described as a heterotrimer of one MRP-8 non-covalently associated with two MRP-14 proteins (Dale et al., 1983), suggesting that this could be the source of the MRP-14. Recently, it has been demonstrated that MRP-14 alone and MRP-8/14 (but not MRP-8 alone) can be secreted from monocytes (Lügering et al., 1997).

1.4.5.2 MRP-8 AND MRP-14 AND CELL MIGRATION

The chemotactic function of MRP-8, described above, is restricted to the murine homologue (CP-10) as human MRP-8 has subsequently been tested and found not to be chemotactic ((Lackmann et al., 1993) and unpublished observations). Despite this lack of activity for human MRP-8, MRP-8 and MRP-14 had previously been implicated in cell migration due to their isolation in complex with macrophage migration inhibitory factor (MIF) (Burmeister et al., 1986). Further implication of their role in cell migration was inferred from the observation that the carboxy-terminal 26 amino acids of MRP-14 were identical to a peptide sequence with neutrophil immobilising factor (NIF) activity (Freemont et al., 1989; Watt et al., 1983). To date, the corresponding peptide from MRP-14 has not been shown to have neutrophil immobilising activity, nor has the MRP-8/14 complex been shown to be chemotactic (Hessian et al., 1993).

One of the most striking observations implicating MRP-8 and MRP-14 in leukocyte migration comes from immunohistochemical studies, in which MRP-8/14 was found associated with vascular endothelium adjacent to marginating neutrophils and monocytes ((Hogg et al., 1989) and **Figure 1.7**). An attractive hypothesis would be that MRP-8 and MRP-14 are released onto vascular endothelium by transmigrating myeloid cells. Localised here, the proteins could then recruit additional or specific populations of leukocytes. The observation that MRP-8/14 is also deposited onto the cuticle surrounding adult worms in nodules of patients with onchocerciasis is reminiscent of the immunohistochemical data (Edgeworth et al., 1993), and may represent a localised concentration of MRP-8/14 for a similar function.

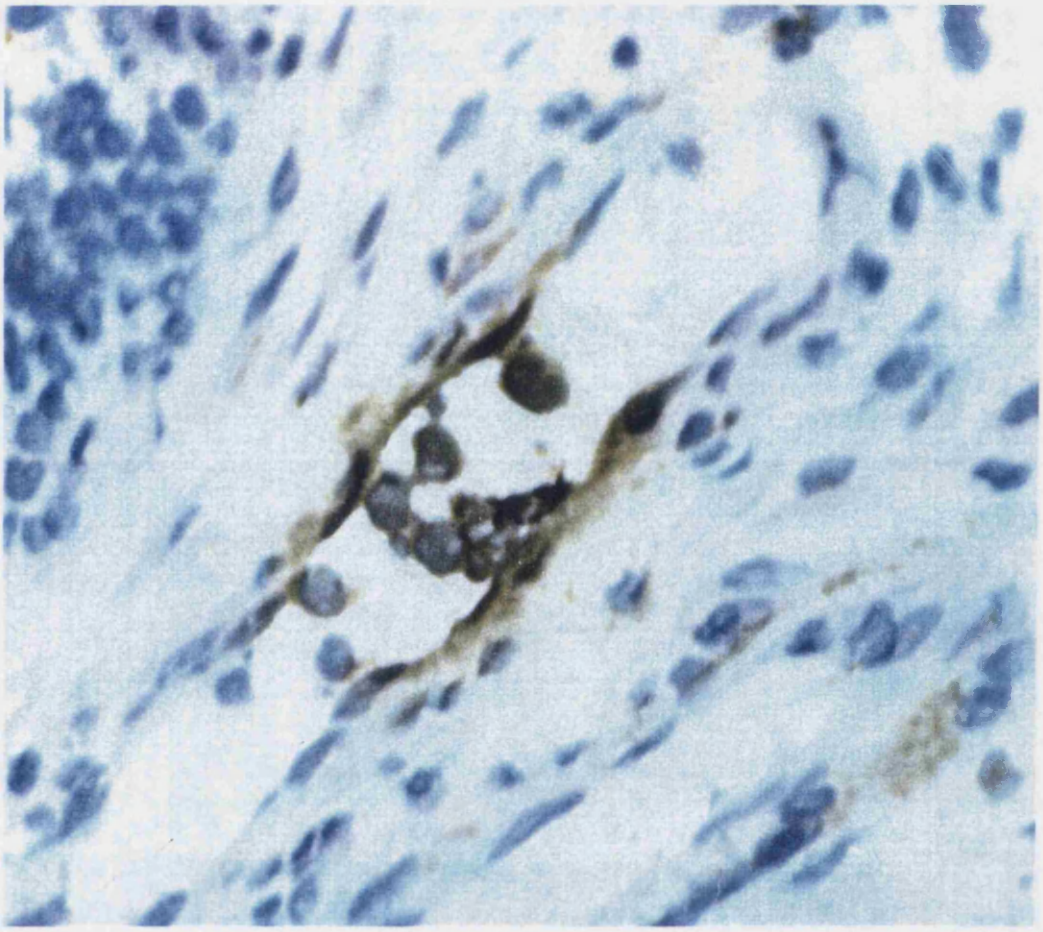

Figure 1.7: Immunoperoxidase staining of a section of rheumatoid synovium. MRP-8/14 is observed deposited on the surface of the endothelium associated with the MRP-8/14-positive myeloid cells. Positive areas of MRP-8/14 protein are detected using mAb 5.5 and are represented by the brown staining.

1.4.5.3 CYTOSTATIC/CYTOTOXIC FUNCTIONS FOR MRP-8/14

An alternate explanation, however, for the localisation of MRP-8/14 in onchocerciasis nodules is that MRP-8/14 are cytostatic or cytotoxic to the infectious agent. Such a speculation comes from the observations that MRP-8/14 are able to cause growth suppression of bacteria and fungi and even some myeloid cell lines (McNamara et al., 1988; Murao et al., 1990; Steinbakk et al., 1990). In addition, expression of MRP-8/14 is the hallmark of necrotic lesions in rheumatoid nodules (Palmer et al., 1987) and it has been suggested that secretion of the complex by infiltrating myeloid cells may initiate the lesion. This is in contrast to the findings of Brun *et al.* (Brun et al., 1995), in which MRP-8/14 was able to protect rats from avridin-induced rheumatoid arthritis. The potential dual functions of MRP-8 and MRP-14 as pro-inflammatory and anti-inflammatory molecules is similar to the duality seen for S100B and may represent a common theme for S100 protein function, depending on the concentration found in the extracellular environment. Extracellular S100B dimer was demonstrated, *in vivo* and *in vitro*, to stimulate astrocytosis and neurite outgrowth (Reeves et al., 1994; Zimmer et al., 1995), proliferation of melanoma cells and induction of apoptosis in PC12 pheochromocytoma cells (Zimmer et al., 1995). The induction of apoptosis occurs at micromolar concentrations, whereas the proliferative functions occur at nanomolar concentrations (Zimmer et al., 1995). Similarly, CP-10 (murine MRP-8) is chemotactic at picomolar levels and MRP-8/14 is cytostatic/cytotoxic at nanomolar levels (Steinbakk et al., 1990). A recent paper has suggested that the ability of MRP-8/14 to induce apoptosis is due to the ability of the protein complex to chelate zinc ions, as the cytotoxic effects of MRP-8/14 were inhibited by 10-50 μM Zn^{2+} ions (Yui et al., 1997; Yui et al., 1995). Similarly, the antimicrobial and candidastatic effects of MRP-8/14 were also attributed to zinc chelation (Murthy et al., 1993; Steinbakk et al., 1990). MRP-14 has recently been demonstrated to bind Zn^{2+} (Raftery et al., 1996); therefore, it would seem likely that the cytotoxic/cytostatic effects of MRP-8/14 are mediated by MRP-14 as MRP-8 does not similarly bind Zn^{2+}. However, the fact that the cytostatic/cytotoxic function is mediated by zinc chelation does raise doubts over the specificity of this MRP-14 function.

1.4.5.4 AN ALTERNATIVE FUNCTION FOR MRP-14?

MRP-14 is the largest of all S100 proteins identified, with an extended C-terminal sequence. The residues 89-114 have recently been demonstrated to be homologous to a sequences within the plasma protein, high molecular weight kininogen (HMWK). These sequences (known as the 'contact domain') within domain 5 of HMWK are

responsible for the binding to the protein to negatively charged surfaces, such as kaolin (Kunapuli et al., 1993). This surface is used to mimic the anionic charge of procoagulant endothelium. MRP-14 similarly binds to kaolin (Hessian et al., 1995), and suggests that this may be a mechanism for MRP-14 or MRP-8/14 retention on the vascular endothelium seen in **Figure 1.7,** although this remains to be tested. MRP-14 lacks other functional procoagulant domains found in HMWK and it was, therefore, speculated that MRP-14 may play a regulatory role in coagulation, perhaps by inhibiting fibrin deposition. In this way, MRP-14 may promote leukocyte migration as fibrin deposition is known to inhibit leukocyte transmigration through endothelium (Kirchofer et al., 1993). Domain 5 of HMWK is also responsible for HMWK binding to other proteins such as heparin (Bjork et al., 1989), Mac-1 (Wachtfogel et al., 1994), and provides part of a composite site recognised by endothelium (Reddigari et al., 1993), platelets (Meloni et al., 1992), and neutrophils (Gustafson et al., 1989). It remains to be determined whether the homologous region MRP-14 is similarly able to bind these cells and what the functional consequences are. Nevertheless, the functional similarity between HMWK and MRP-14 further implicates MRP-14 in the extravasation of leukocytes.

1.4.5.5 SIGNALS IN RESPONSE TO S100 PROTEINS IN THE EXTRACELLULAR ENVIRONMENT

Both the neurite outgrowth and the apoptosis induced by S100B are characterised by an increase in $[Ca^{2+}]_i$ (Barger and Van Eldik, 1992; Fano et al., 1993), suggesting that S100B is able to elicit intracellular signals in the target cell. The response of cells to CP-10 is in contrast to the response to S100B, as no increase in $[Ca^{2+}]_i$ is observed (Cornish et al., 1996). This also further contrasts CP-10 from the chemokines and other classical chemoattractants, which are characterised by their ability, among other things, to induce $[Ca^{2+}]_i$ mobilisation (reviewed in (Baggiolini et al., 1994)). The other two chemotactic S100 proteins have not been tested for their ability to increase $[Ca^{2+}]_i$, but at least S100L exhibits functional similarities to CP-10 as the response of cells to both proteins is mediated by the signals emanating from G protein-coupled receptors. No receptor, however, has been identified for S100B or the other S100 proteins functional in the extracellular environment. Future studies will determine whether these S100 proteins are all operating through G protein-coupled receptors, or whether there are different receptors for different subsets within the family.

1.5 MAJOR AIM OF THE THESIS

The major aim of this thesis was to investigate potential extracellular functions for MRP-8 and MRP-14. Based on the evidence presented above, the role of MRP-8 and MRP-14 in leukocyte extravasation was investigated. This involved investigating the function of MRP-8 and MRP-14 in both myeloid cell and lymphocyte integrin adhesion and migration, using *in vitro* assay systems.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 INHIBITORS AND STIMULI

REAGENT	STOCK CONCENTRATION	SUPPLIER
BCECF/AM (2', 7-bis (carboxyethyl)-5 (6')-carboxy fluorescein pentaacetoxymethyl) ester)	1mM in DMSO	Calbiochem, Nottingham UK
bis-INDOLYLMALEIMIDE	10mM in DMSO	Sigma, Poole UK
di-HYDROCYTOCHALASIN B	5mg/ml in DMSO	Sigma
CYTOCHALASIN D	1mg/ml in DMSO	Sigma
di-HYDRORHODAMINE 123	30mM in DMSO	Cambridge Bioscience, Cambridge UK
fMLP (formyl-leucyl-methionyl-phenylalanine)	10mM in ethanol	Sigma
FURA-2/AM {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethyl) ester}	1mM in DMSO	Calbiochem
HERBIMYCIN A	1mM in DMSO	GIBCO BRL Life Techn., Paisley UK
LY-294002	100mM in DMSO	Calbiochem
PD098059	30mM in DMSO	Calbiochem
PMA (Phorbol-12-myristate-13 acetate)	2mM in DMSO	Calbiochem
WORTMANNIN	10mM in DMSO	Calbiochem

2.1.2 BUFFERS

PBS-A (1x and 10x) and RPMI-1640 buffers were supplied autoclaved from the ICRF Central Cell Services. Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS; 10x), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES, 1M) and RPMI-1640, all low endotoxin, were purchased from Sigma. Buffer constituents are described in Appendix I.

2.1.3 RECOMBINANT PROTEINS

Recombinant S100A and S100B (Sigma) were reconstituted with water to 2mg/ml. Interleukin (IL)-8 (Sigma) was reconstituted with water to 200μg/ml. All proteins were stored at 4°C for the short term or -70°C for the long term.

2.1.4 MONOCLONAL ANTIBODIES

The following mAbs were used in this study at the following concentrations unless otherwise stated:

MAb	ISOTYPE	EPITOPE	CONCENTRATION USED	SUPPLIER
14	IgG1	CD8	neat supernatant	Leukocyte Adhesion Laboratory (LAL), ICRF
2H3	IgG1	Mac-1 α subunit (CD11b)	1/100 ascites	Leukocyte Typing VI Workshop
2LPM19c	IgG1	Mac-1 α subunit (CD11b)	20μg/ml purified or 1/50 ascites	Dr. Karen Pulford, Oxford
24	IgG1	β_2 integrin activation reporter	10μg/ml purified	LAL
27E10	IgG1	MRP-8/14 (complex specific)	1/50 purified mAb	BMA Biomedicals AG, Switzerland
38	IgG2a	LFA-1 α subunit (CD11a)	10μg/ml purified	LAL
3.9	IgG1	p150,95 α subunit (CD11c)	10μg/ml purified	LAL
4U	IgG2a	isotype control	10μg/ml purified	LAL
52U	IgG1	isotype control	10μg/ml purified	LAL
HP1/2	IgG1	VLA-4 α subunit (CD49d)	3μg/ml purified	Dr. Roy Lobb, Biogen Inc., Cambridge MA
ICRF44	IgG1	Mac-1 α subunit (CD11b)	10μg/ml purified	LAL
ICRF44-FITC	as above	as above	4μg/ml purified	Sigma

LAM1.3	IgG1	L-selectin (CD62L)	5µg/ml purified	Dr. Tom Tedder, Dept. of Immunology, Duke University, North Carolina
MEM 25	IgG1	LFA-1 α subunit (CD11a)	1/50 ascitic fluid	Dr. Vaclav Horesji, Institute of Medical Genetics, Prague
MEM 56	IgG1	CD45RA	10µg/ml purified	Dr. Vaclav Horesji
P5D2	IgG1	β_1 subunit (CD29)	10µg/ml purified	Developmental Studies Hybridoma Bank, Iowa
SAM-1	IgG2b	VLA-5 α subunit (CD49e)	1µg/ml purified	Eurogenetics, UK
UCHT1	IgG1	CD3	neat supernatant	Prof. Peter Beverley, UCL
UCHL1	IgG1	CD45R0	10µg/ml purified	Prof. Peter Beverley

2.2 METHODS

2.2.1 ESTIMATION OF PROTEIN CONCENTRATION

Protein concentrations were estimated using the Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) in 96 well microtitre plates. Gamma globulin standards from 0-500 µg/ml were used to calibrate the estimations. Briefly, 5µl of either test or standard protein were added in triplicate to wells together with 250µl of diluted (1/5) dye reagent concentrate. The plate was allowed to incubate at RT for 5-15 min prior to reading the OD at 595nm using a Titertek Multiskan plate reader (Titertek, UK). Test protein concentrations were calculated from the standard curve of protein concentration of gamma globulin standards versus OD readings.

2.2.2 FLUORESCEIN ISOTHIOCYANATE (FITC) LABELLING OF PROTEINS

FITC labelling of MRP-14, fibrinogen and mAbs was modified from the method of Goding (Goding, 1986). Briefly, human plasma fibrinogen (2mg/ml; Sigma) was dissolved in 0.1M carbonate buffer, pH 9.5 and MRP-14 (2.5mg/ml) and mAbs (2-4 mg/ml) were dialysed overnight into the same buffer. FITC (10mg/ml in DMSO; Sigma) was added to the protein solutions to a final concentration of 25µg/ml and labelling was allowed to proceed at room temperature for 1-3 hours. Unbound FITC was removed by gel filtration in either HBSS containing 10mM HEPES (H-HBSS), pH 7.5 (MRP-14) or PBS (fibrinogen and mAbs) using a pre-equilibrated PD-10

column (Pharmacia Biotech, St. Albans, UK). Optical densities at 280 and 495 nm were measured and F/P ratio was determined by spectrometry and the fluorescence/protein (F/P) ratios were calculated according to the following equation:

$$F/P = \frac{2.87 \times OD_{495}}{OD_{280} - (0.35 \times OD_{495})}$$

Labelled fibrinogen at 1mg/ml with an F/P ratio of between 2-3, MRP-14 at 1.3mg/ml with an F/P=6 and mAb 24 at 2mg/ml with an F/P=3.4, were used. FITC-conjugated mAb ICRF44 was purchased from Sigma and was supplied at 200µg/ml with an F/P=4.7.

2.2.3 PURIFICATION OF MRP-8/14 HETERODIMER

MRP-8/14 heterodimer was purified from neutrophil or chronic granulocytic leukaemia (CGL) leukocyte cytosol by Fast Protein Liquid Chromatography (FPLC), as previously described (Edgeworth et al., 1991). Neutrophils isolated from 500ml of blood or a 50ml leukocyte pellet from a CGL patient were washed at 4°C twice in PBS and 2x in 20mM Tris-HCl, pH 8.0, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol (DTT; Sigma) (Mono Q buffer). Cells were then lysed by sonication on ice and cytosolic protein was separated from cell membranes by centrifugation for 30 min at 4°C at 17000 *g*. Cytosolic protein was then dialysed overnight at 4°C in 1000 molecular weight cut-off dialysis tubing (Pierce & Warriner, Chester, UK) against Mono Q buffer. Prior to loading on the Mono Q anion-exchange column, the protein solution was centrifuged again at 17000 *g* for 1 hour, followed by ultracentrifugation at 55000 *g* for 1 hour (both at 4°C). The supernatant was then injected onto the Mono Q column, pre-equilibrated with 5 volumes of Mono Q buffer. A maximum of 10mg of total protein was loaded in any one run. Bound protein was eluted from the column by the application of a 0-1M NaCl gradient in Mono Q buffer over 30 min at a flow rate of 1ml/min. Fractions containing MRP-8/14, as determined by 15% SDS-PAGE were either dialysed directly into PBS-A (neutrophil preparations) or diluted 1/5 (CGL preparations) in 25mM sodium acetate, pH 4.5, 0.15M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT (Mono S buffer). Diluted MRP-8/14 fractions in Mono S buffer were then injected onto a pre-equilibrated Mono S cation-exchange column at 1ml/min and bound protein was eluted by the application of a 0.15-1M NaCl gradient in Mono S buffer over 30 min. Fractions containing MRP-8/14 were again determined by 15% SDS-PAGE and dialysed into PBS-A. All preparations were stored at 4°C at ~2mg/ml.

2.2.4 PREPARATION OF RECOMBINANT MRP-8 AND MRP-14

Recombinant human MRP-14 and MRP-8 proteins were prepared from *E. coli* BL21 (DE3) plysS bacteria transformed with pET3a expression vectors containing the cDNAs for these proteins constructed by Dr. Paul Hessian in the Leukocyte Adhesion Laboratory. The methods were optimised by the ICRF Protein Isolation and Cloning Laboratory. Recombinant MRP-14 was purified to homogeneity using a 2 step procedure. The bacterial pellet from 1 litre of overnight culture was sonicated into 40ml of 50mM Tris pH 8, 2mM EDTA, 1mM DTT and 2mM PMSF. A further 40ml of buffer was added, together with 50μg/ml DNAse and 10mM $MgCl_2$. The solution was then centrifuged for 20 min at 12000 *g* and 4°C. The pellets were resuspended in 80ml of buffer and re-centrifuged twice as before. The final pellets were resuspended in 10ml 100mM Tris-HCl, pH 8 and 30ml of 8M urea was added. This solution was incubated on ice for 1 hour and then dialysed overnight against Mono P buffer A (25mM bis-Tris, pH 7.1). The dialysed extract was centrifuged at 150000 *g* for 30 min at 4°C and then applied to a Mono P chromatofocussing column, prepared and run according to the manufacturer's instructions (Pharmacia Biotech). Bound MRP-14 was eluted with buffer B (10% Poly buffer 76, pH 4; Pharmacia Biotech). Fractions containing MRP-14, as determined by SDS-PAGE, were dialysed into 5mM Na_2HPO_4, 0.1M NaCl, pH 8 (buffer A) and applied to a hydroxyapatite column (Bio-Rad). The column was equilibrate with 1:1 ratio of buffer A and buffer B (500mM Na_2HPO_4, 0.1M NaCl, pH 8). Bound MRP-14 was eluted by a linear gradient of 5-500mM Na_2HPO_4. Fractions containing MRP-14 were pooled, concentrated to 5mg/ml in H-HBSS and stored at -70°C. Recombinant MRP-8 was similarly purified by a 2 step procedure. In the first step, bacterial extract (prepared as for MRP-14) was applied to a Rotofor Preparative Focussing Cell (Bio-Rad), according to manufacturer's instructions. The second step was identical to that for MRP-14 purification. Purified MRP-8 was stored at 2mg/ml in H-HBSS at -70°C.

2.2.5 REDUCTION AND ALKYLATION OF RECOMBINANT MRP-8

Recombinant MRP-8 was expressed in multimeric forms and was reduced and alkylated prior to use as a monomer (Stanworth and Turner, 1986). MRP-8 at 2mg/ml was dialysed into Tris-HCl, pH 8 and DTT was added to a final concentration of 0.02M. The mixture was incubated at RT for 30 min before iodoacetamide (Sigma) was added (final concentration 0.12M) and the mixture incubated for a further hour. Iodoacetamide and DTT were removed by gel filtration using a PD-10 column (Pharmacia Biotech) and the protein solution was dialysed back into H-HBSS.

2.2.6 ENDOTOXIN REMOVAL FROM MRP-14 PROTEIN SOLUTIONS

Endotoxin was removed from MRP-14 solutions by phase separation with Triton X-114 (Sigma), according to the method of Aida and Pabst (Aida and Pabst, 1990). Protein solutions were vortexed in the presence of 1% Triton X-114 and then placed on ice for 5 min. After this time the samples were vortexed again and warmed to 37°C for 5 min to allow two phases to form. Samples were then centrifuged for 7 seconds in a microfuge and the upper aqueous phase (containing the protein) was removed. To eliminate the possibility of detergent contamination, the procedure was repeated without further addition of detergent. Buffer controls were also prepared using this procedure. Endotoxin levels were determined in all samples using the *Limulus* amoebocyte lysate kit (Sigma), according to the manufacturer's instructions. Dilutions of test samples were incubated with *Limulus* lysate (E-toxate, Sigma) at 37°C for 60 min. Test samples were compared to endotoxin standards and the level of positive contamination was judged by the highest dilution of the sample that formed a gel.

2.2.7 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli *et al.*(Laemmli, 1970). A 15% polyacrylamide gel (stock 30%w/v acrylamide/0.8%w/v bis-acrylamide; Anachem, UK) in 375mM Tris, pH 8.8, 0.1% SDS, 0.04% ammonium persulphate (APS) and 1/500 N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma) was used to separate low molecular weight proteins. This was overlaid with a 3%polyacrylamide stacking gel which was composed of 125mM Tris, pH 6.8 and 0.1% SDS and polymerised as above with APS and TEMED. Proteins were boiled for 5 min in sample buffer (125mM Tris, pH6.8, 4% SDS, 27.5% glycerol, 0.002% bromophenol blue). Reducing samples contained, in addition, 1% 2-mercaptoethanol (Sigma). Low molecular weight rainbow markers (Amersham, UK) covering a range of 2.5-46 kDa were prepared in the same way as protein samples. Samples were subjected to electrophoresis in 25mM Tris/192mM glycine, pH 8.3, 0.1% SDS at 100V until samples reach separating gel at which time the voltage is increased to 180V. Proteins were visualised by staining with Coomassie Blue (0.5% w/v Coomassie Blue, 40% ethanol, 10% glacial acetic acid) and destained with 20% ethanol/10% glacial acetic acid.

2.2.8 ELISA

Nunc-Immuno™ Maxisorp™ 96 well plates (GIBCO BRL) were incubated overnight at 4°C with 2μM of MRP-14 or MRP-8 alone, or together in H-HBSS/ 2mM Ca^{2+}/2mM Mg^{2+}/20μM Zn^{2+} (50μl/well). Following incubation, the liquid was aspirated and the plate was blocked with 150μl/well of PBS/0.1% Tween 20 for 1 h at RT. The plate was washed 3x and incubated sequentially (including washes) with 50μl/well of the MRP-8/14 complex-specific mAb 27E10 (Bhardwaj et al., 1992) (1/50 in PBS/0.1% Tween 20) for 1 h at RT, followed by 30 min at RT with peroxidase-conjugated goat anti-mouse Ig (1/2000; DAKO, High Wycombe, UK) in PBS/Tween. Bound Ig was detected using O-phenylenediamine dihydrochloride (OPD; Sigma) according to the manufacturer's instructions.

2.2.9 CELL CULTURE

T lymphoblasts were expanded from peripheral blood mononuclear cells obtained by centrifugation (600*g*) of buffy coat cells over Lymphoprep® (Sodium metrozoate/Ficoll solution; Nycomed, Sweden) and then washed three times in RPMI-1640 (ICRF, Media Supplies). Cells were resuspended in RPMI-1640 containing 10% FCS (Sigma) and 1μg/ml phytohaemagglutinin (PHA; Murex Diagnostics, UK) and cultured for 4 days at 37°C in a 5% CO_2 humidifier. Cells were then washed out of PHA and into RPMI-1640/10%FCS, containing 20ng/ml Interleukin-2 (IL-2; Eurocetus, USA) T lymphoblasts were then washed every 2 days into double the volume of RPMI/FCS/IL-2 and used between days 8 and 14.

Mac-1-transfected (KC/16) and untransfected K562 erythroleukaemic cells were maintained at 37°C and 5% CO_2 in DMEM (ICRF) supplemented with 10% FCS (Ortlepp et al., 1995). Geneticin G418 (0.5mg/ml; GIBCO BRL, Paisley, UK) was added to Mac-1 transfected cell media to maintain selection.

2.2.10 ISOLATION OF POLYMORPHONUCLEAR LEUKOCYTES (NEUTROPHILS)

Human neutrophils were isolated from EDTA-anticoagulated venous blood from normal healthy donors by dextran sedimentation and density gradient centrifugation (Dooley et al., 1982). Briefly, erythrocytes sedimentation was expedited by the addition of dextran T500 (Pharmacia) to a final concentration of 0.6%. After 30-60 min at RT the leukocyte-rich fraction was loaded onto a discontinuous (70%/80%)

isotonic Percoll (Pharmacia) gradient and centrifuged at 800 *g* for 15 min at RT. The neutrophil fraction was removed from the 70% and 80% Percoll gradient interface and washed 3x in H-HBSS and resuspended at 5 x 10^6/ml. Neutrophils were >95% pure, as judged by morphology; and >98% viable, as determined by Trypan Blue dye exclusion (Sigma).

2.2.11 NEUTROPHIL ADHESION ASSAYS

Nunc-Immuno™ Maxisorp™ plates (GIBCO BRL) were coated overnight with 50µl/well 2mg/ml fibrinogen in 0.1M carbonate buffer (Sigma). Similarly, Immulon-1 96 well plates (Dynatech, USA) were coated with 6µg/ml ICAM-1Fc in PBS. ICAM-1-coated plates were blocked with 1% gelatin (Sigma) for 2 hours at 37°C. All plates were washed 3 times in assay buffers to remove unbound protein. Purified neutrophils ($5x10^6$/ml) were labelled with 1µM of the intracellular fluorescent dye, BCECF/AM (Calbiochem) for 30 min at room temperature (RT). After three washes, labelled cells were resuspended in H-HBSS at $2x10^6$/ml and 50µl were added to the protein-coated wells. These wells contained an equal volume of H-HBSS with 2x cations (2mM $CaCl_2$, 2mM $MgSO_4$, 20µM $ZnSO_4$) together with stimulating or blocking reagents. The plate was allowed to incubate at RT (fibrinogen) or 37°C (ICAM-1) for 30 min and, after washing 2-4 times with H-HBSS, the resultant adherent cells were quantitated using a 96 well plate fluorescence reader (Fluoroskan II; Labsystems, Basingstoke, UK). Percentages of adherent cells were determined using fluorescence values of 50µl of total cells added per well. For the pertussis toxin inhibition experiments, cells were preincubated with various concentrations of *Bordetella pertussis* toxin (Sigma) for 30 minutes at room temperature.

2.2.12 T CELL ADHESION ASSAYS

T cell adhesion was performed essentially as for neutrophils, with the following exceptions and additions: Plates were also coated with 20µg/ml fibronectin (Sigma) as for ICAM-1Fc. T cells were washed 3 times out of RPMI/FCS/IL-2 into H-HBSS and labelled with 2µM BCECF/AM for 30 min at 37°C. ICAM-1Fc and fibronectin assays were performed at 37°C. For antibody inhibition of fibronectin binding, T cells were preincubated for 15 min on ice, prior to 30 min at 37°C.

2.2.13 MEASUREMENT OF INDIRECT IMMUNOFLUORESCENCE USING FLOW CYTOMETRY

Purified neutrophils ($5x10^5$/sample) were incubated at RT in H-HBSS containing 1mM Ca^{2+}, 1mM Mg^{2+} and 10μM Zn^{2+}, in the presence or absence of MRP-14 or fMLP, together with primary mAbs at concentrations stated. After 30 min the cells were washed 3 times with PBS/0.2% BSA/0.1% azide (FACSwash) and resuspended in FITC-conjugated goat anti-mouse IgG (1/200; Sigma). After 30 min on ice, the cells were again washed 3 times and resuspended in 200μl FACSwash. The fluorescence intensity was then determined using a FACScan flow cytometer (Becton Dickinson). For T cells, indirect immunofluorescence was performed as above with the following exceptions: cells were either incubated on ice or at 37°C, as indicated in the Figure legends, instead of RT; Mn^{2+} was used as control stimulus instead of fMLP; and for detection of mAb SAM-1 (anti-VLA-5), goat anti-mouse IgG2b (1/200; Southern Biotechnology Assoc. Inc., USA) was used.

2.2.14 FLOW CYTOMETRY FOR sICAM-1 BINDING

Measurement of soluble ICAM-1Fc binding to T cells was based on the method of Stewart *et al.* (Stewart et al., 1996). T cells were washed 3 times into H-HBSS and resuspended at 4 x 10^6/ml. Aliquots (50μl) of cells were then added to Flexiwells (Dynatech) containing 50μl of double concentrations of stimulants, cations, and indicated concentrations of ICAM-1Fc. After 30 min at 37°C, cells were washed 3 times in ice cold FACSwash and then incubated with 10mg/ml FITC-conjugated goat anti-human IgG Fc specific antibody (Jackson Immunoresearch Laboratories, USA) for 30 min on ice. Unbound secondary antibody was removed by washing 3 times in ice-cold FACSwash and the FL-1 fluorescence was detected as previously described.

2.2.15 MEASUREMENT OF DIRECT IMMUNOFLUORESCENCE OF FITC-CONJUGATED PROTEINS AND mAbs

In experiments measuring soluble FITC-fibrinogen binding 24-FITC (β_2 integrin activation reporter mAb) or ICRF44-FITC (anti-Mac-1 α subunit (CD11b)), the FITC-fibrinogen and FITC-mAbs replaced the primary antibody and after 30 min at RT (neutrophils) or 37°C (T cells), the cells were washed 3 times, resuspended in FACSwash and analysed as above. To test for cation dependence, cells were incubated

with FITC-fibrinogen and MRP-14 in H-HBSS without Ca^{2+}, Mg^{2+} and Zn^{2+}. Control stimuli were used as for indirect immunofluorescence. In experiments measuring Mac-1 expression in whole blood, 10µl of FITC-conjugated mAb 44 was added to 100µl of whole blood. After 30 min at RT cells were placed on ice and red blood cells lysed by the addition of lysis buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.1M Na_2EDTA) for 5 min. Cells were then washed 3 times with ice-cold FACSwash and analysed as above. Channel 1 fluorescence of neutrophils was measured by gating the population based on their forward and side scatter.

2.2.16 MEASUREMENT OF DIRECTLY CONJUGATED MRP-14 BINDING TO CELLS

In experiments measuring FITC-MRP-14 binding, neutrophils or K562 cells ($1x10^5$/sample) were incubated on ice in H-HBSS containing 1mM Ca^{2+}, 1mM Mg^{2+}, 10µM Zn^{2+} and 0.1% BSA (incubation buffer) with varying amounts of FITC-MRP-14. After 30 min, the cells were washed 3 times and resuspended in 200µl ice cold incubation buffer. To test for specific binding, increasing amounts of unlabelled MRP-14 and a control S100 protein (S100A) were incubated, as above, with 2µM FITC-MRP-14.

2.2.17 MEASUREMENT OF β-GLUCURONIDASE RELEASE

Neutrophils ($5 x10^6$/ml) were preincubated in individual polypropylene tubes (Nunc Minisorp, GIBCO BRL) for 5 min at 37°C with 5µg/ml dihydrocytochalasin B (Sigma) in H-HBSS with 1mM Ca^{2+}, 1mM $Mg^{2+}$and 10µM Zn^{2+}. After this time, stimuli were added and the cells incubated for a further 30 min at 37°C. Cells were then centrifuged and the supernatant transferred to 1.5ml Eppendorf tubes (Sarstedt, Numbrecht, Germany). A 200µl aliquot of supernatant was transferred to a 10ml polypropylene tube (Nunc, GIBCO BRL) and β-glucuronidase release was measured as per kit instructions (Sigma). Briefly, 600µl 0.2M acetate buffer, pH 4.5, was added to the supernatant, together with 200µl of 30mM phenolphthalein mono-β-glucuronic acid, pH 4.5. The contents of the tubes were mixed and incubated in a 56°C water bath for 1 hour. Immediately following incubation, 5ml of 2-amino-2-methyl-1-propanol (AMP) buffer was added to each tube. The absorbance at 550nm of each tube was measured and compared to controls without cell supernatant. Results were

converted to ng/ml of phenolphthalein using a calibration curve of the absorbance of 550nm of known phenolphthalein concentrations, according to manufacturer's instructions.

2.2.18 MEASUREMENT OF NEUTROPHIL INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using FURA-2/AM according to the method of Tsien *et al.* (Tsien et al., 1982). Cells ($5x10^6$/ml) were incubated with 1μM Fura-2/AM (Calbiochem) in H-HBSS for 30 min at RT. After 3 washes, the cells were resuspended at 1x 10^6/ml in H-HBSS containing 1mM Ca^{2+}, 1mM $Mg^{2+}$and 10μM Zn^{2+}. Aliquots of cells (2 ml) were placed in quartz fluorimeter cuvettes (Sigma) and allowed to warm to 37°C with constant stirring. Stimulants were added at indicated time points. Fluorescence was monitored over 7 min using a Perkin-Elmer LS-5 luminescence spectrophotometer (Perkin-Elmer, Beaconsfield, UK) with an excitation wavelength of 336nm and an emission wavelength of 510nm.

2.2.19 NEUTROPHIL RESPIRATORY BURST AS MEASURED BY SUPEROXIDE PRODUCTION

The production of superoxide was measured according to the method of Smith and Weidemann (Smith and Weidemann, 1993). Briefly, purified neutrophils ($2x10^6$/ml in HBSS) were labelled with 1μM di-hydrorhodamine (DHR) 123 (Cambridge Bioscience, Cambridge, UK) for 5 min at RT prior to exposure to various stimuli. After 30 min stimulation at RT, cells were immediately placed on ice and their channel 1 fluorescence intensity measured by flow cytometry using a FACScan.

2.2.20 CHEMOTAXIS ASSAY

Dilutions of MRP-14 in H-HBSS containing 1mM Ca^{2+}, 1mM Mg^{2+} and 10μM Zn^{2+} and 0.1% BSA (assay medium) were added to Costar 24-well tissue culture plates (GIBCO BRL) in a final volume of 600μl in triplicate. Costar Transwells (6.5 mm diameter; 3μM pore size; GIBCO BRL) were precoated on both sides with 50μg/ml fibrinogen (Sigma) overnight at 4°C. The Transwells were washed twice with H-HBSS and once with assay medium before insertion into assay wells. Either 100 μl of

BCECF/AM-labelled neutrophils ($5x10^6$/ml) in assay medium alone or containing 1μM of MRP-14 were added to the top chamber. Cells were allowed to transmigrate for 1 h at 37°C. After this time, Transwells were removed and the liquid remaining on the underside of the inserts was pipetted into the wells. Any migrated cells which had adhered to the bottom of the well were detached using 5mM EDTA and counted using a flow cytometer (Becton Dickinson).

2.2.21 CONFOCAL MICROSCOPY

For immunofluorescence analysis of F-actin staining, 13mm glass coverslips were precoated with 2mg/ml fibrinogen, overnight at 4°C and then washed 4 times with H-HBSS. Buffer conditions were as for the 96 well plate adhesion assay. Unlabelled neutrophils ($4x10^5$) were added to each coverslip in the presence or absence of stimulants in a total volume of 400μl. Coverslips were spun at 40g then incubated for 30 min at RT. Non-adherent cells were removed by gentle washing in H-HBSS. Cells were fixed, permeabilised and stained for 30 min on ice by the addition of H-HBSS/1% formaldehyde/0.2% Triton-X 100/0.25μg/ml FITC-phalloidin. Coverslips were then washed 4 times and mounted on slides. Confocal microscopy was performed using a Leica TCS NT microscope equipped with a 60 x oil immersion objective (Leica, Wetzlar, Germany).

2.2.22 NEUTROPHIL F-ACTIN MEASUREMENTS

FITC-phalloidin was used to measure filamentous actin (F-actin) according to the method of Cornish *et al.* (Cornish et al., 1996). Briefly, neutrophils were pre-warmed in H-HBSS with 1mM Ca^{2+}, 1mM Mg^{2+} and 10μM Zn^{2+} for 5 min at 37°C in 1.5 ml polypropylene tubes (Sarstedt). After this time, stimuli were added and cells further incubated for various time points up to 3 min. Immediately after incubation, the tubes were placed on ice and fixed and permeabilised by the addition of an equal volume of 7.8% formaldehyde/H-HBSS containing 1mM Ca^{2+}, 1mM Mg^{2+}, 10μM Zn^{2+} and 100μg L-α-lysophosphatidyl choline. Tubes were left on ice for a further 60 min on ice with 0.5μM FITC-phalloidin (Sigma) before cells were centrifuged (400 *g*, 10 min) and washed twice with 5 volumes of ice-cold H-HBSS with cations. Flow cytometric analysis was performed as for immunofluorescence.

THE EFFECT OF S100 PROTEINS, MRP-8 AND MRP-14, ON NEUTROPHIL ADHESION

3.1 INTRODUCTION

MRP-8 and MRP-14 are abundantly expressed as a heterodimeric complex (MRP-8/14) in the cytosol of circulating human myeloid cells. Previous results from our laboratory identified MRP-8/14 deposited on the surface of certain vascular endothelium in both non-inflamed and inflamed tissues (Hessian et al., 1993; Hogg et al., 1989). These experiments employed a monoclonal antibody (mAb 5.5), directed against a common epitope in MRP-8 and MRP-14 (Edgeworth et al., 1991; Hogg et al., 1989; Hogg et al., 1985) to stain tissue sections. Areas of positive MRP-8/14 (mAb 5.5) staining on endothelia were visible associated with adherent MRP-8/14-positive myeloid cells (see **Figure 1.7**). Other areas of the same vessel were mAb 5.5 negative and, even after stimulation with cytokines or phorbol esters, the endothelia themselves did not express MRP-8/14, suggesting the source of MRP-8/14 was the myeloid cells. In addition, myeloid cells which had transmigrated through endothelium were observed to be negative for mAb 5.5 staining (Hogg et al., 1989). Other cytoplasmic proteins were not simultaneously released onto the vessel, indicating that MRP-8/14 was actively secreted from the myeloid cell and was not the product of non-specific cell leakage. It was hypothesised from these observations that extracellular MRP-8/14 deposited onto the surface of the endothelium may play a role in myeloid cell adhesion and transendothelial migration.

MRP-14 has been previously implicated in neutrophil migration as the C-terminal 26 amino acids were identical to a sequence within a peptide with neutrophil immobilising activity (neutrophil immobilizing factor, or NIF) (Freemont et al., 1989; Watt et al., 1983). MRP-14 (or MRP-8/14) could, therefore, potentially participate in immobilising neutrophils on endothelium at sites of extravasation to inflammatory sites. Based on these observations, the role of MRP-8/14 in neutrophil adhesion was investigated.

3.2 RESULTS

3.2.1 THE EFFECT OF THE MRP-8/14 HETERODIMER ON NEUTROPHIL ADHESION TO FIBRINOGEN

Initial experiments were designed to study the effect of the purified native human MRP-8/14 heterodimer on neutrophil adhesion to immobolised fibrinogen. Native human MRP-8/14 complex was purified from the cytosolic protein of whole blood from chronic granulocytic leukaemia (CGL) patients. A combination of anion- and cation-exchange Fast Protein Liquid Chromatography (FPLC) was used according to the method previously described by Edgeworth, *et al.* (Edgeworth et al., 1991) and listed in the Materials and Methods. A single peak corresponding to the heterodimeric complex was eluted from the Mono S cation-exchange column (data not shown). This peak represented mostly non-covalently associated heterodimer as, under non-reducing SDS-PAGE conditions, the MRP-8/14 was dissociated to its subunit proteins (**Figure 3.1A, lane a**). A small proportion of the MRP-8/14 was covalently associated and migrated at ~26 kDa under non-reducing conditions on SDS-PAGE and only dissociated to MRP-8 and MRP-14 under reducing conditions (**Figure 3.1A, lane b**). The faint band at 28 kDa on the non-reducing gel but absent from the reducing gel indicated the presence of a small amount of MRP-14 homodimer (**Figure 3.1A**). The presence of a contaminating bands at 21 and ~55 kDa were not consistently present at the same levels in all preparations (**Figure 3.1A** and data not shown).

In later experiments, the method of MRP-8/14 purification was modified to use purified neutrophil cytosolic protein from a normal healthy donor and only a single round of anion-exchange (Mono Q) chromatography. The MRP-8/14 preparations from neutrophil cytosol were of greater purity after anion-exchange chromatography compared to the CGL preparation after anion- and cation-exchange chromatography (**Figure 3.1B** and data not shown). Both methods yielded milligram quantities, which is in accordance with results published by Edgeworth *et al.* (Edgeworth et al., 1991). Approximately 10 mg of MRP-8/14 were obtained from ~2.5 x 10^9 CGL neutrophils, whereas 5 mg were purified from ~0.5-1 x 10^9 normal neutrophils. The apparent decreased yield from CGL neutrophils was not from differences in the quantity of protein loaded onto the anion-exchange column, but are due to losses in the subsequent chromatographic steps (data not shown). The identities of the proteins in Figure 3.1 were confirmed as MRP-8 and MRP-14 by Western blotting and staining with mAb 5.5, which recognises both the individual proteins and the heterodimeric complex (data not shown).

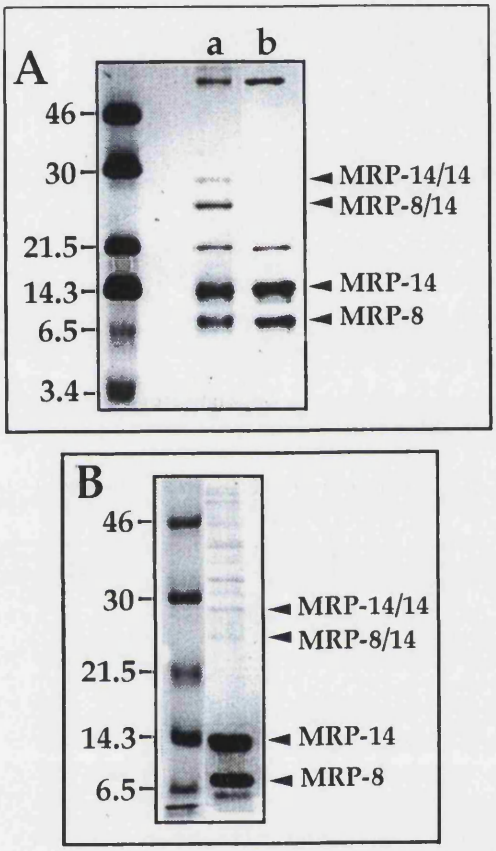

Figure 3.1: Purification of MRP-8/14 heterodimer from the cytosol of human chronic granulocytic leukaemia cells and normal human neutrophils. A. 15% SDS-PAGE of purified MRP-8/14 from CGL cell cytosol under **a)** non-reducing or **b)** reducing conditions. **B.** 15% SDS-PAGE of MRP-8/14 from human neutrophil cytosol under non-reducing conditions. Both gels were stained with Coomassie Blue.

The heterodimeric MRP-8/14 complex in Figure 3.1A was tested, at concentrations of up to 100 μg/ml, for its ability to induce neutrophil adhesion to fibrinogen, compared to the known stimulator fMLP. Unlike fMLP, which stimulated approximately 10% neutrophil adhesion at 0.1μM, MRP-8/14 was unable to induce adhesion at any concentration tested (**Figure 3.2**). In addition, MRP-8/14 did not inhibit or enhance the ability of fMLP to stimulate adhesion (**Figure 3.2**).

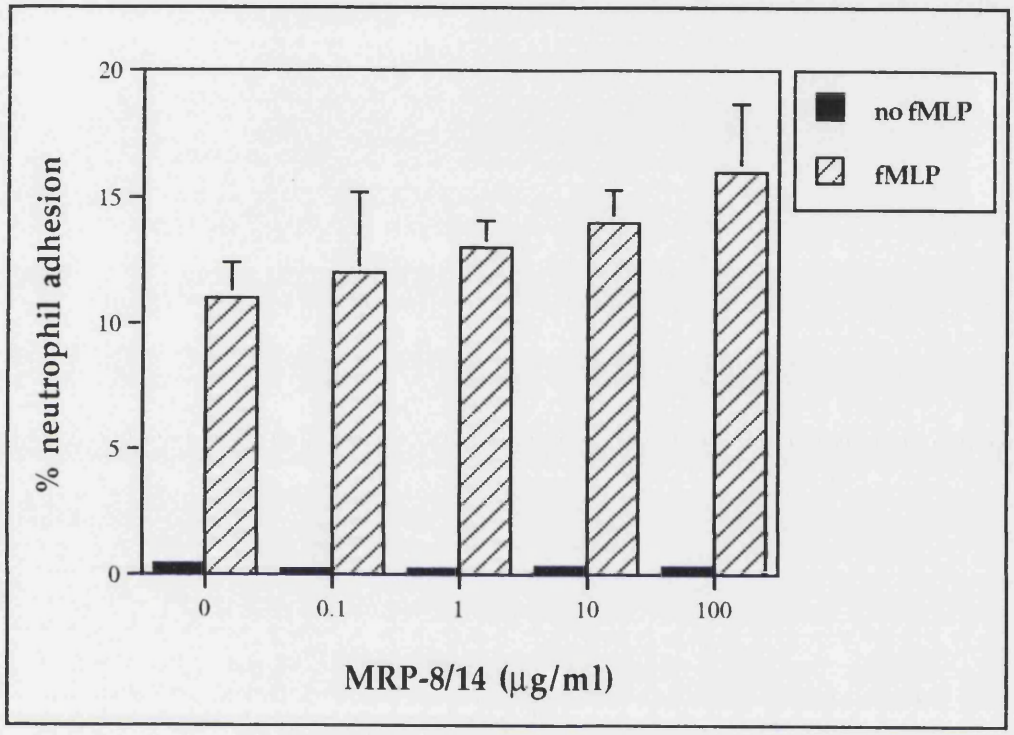

Figure 3.2: MRP-8/14 does not affect neutrophil adhesion to fibrinogen. MRP-8/14 was titrated from 0-100 µg/ml in 96 well plates for the ability to induce human neutrophil adhesion to fibrinogen in the presence (▨) or absence (■) of 0.1µM fMLP. Neutrophils were incubated in the presence of stimuli at room temperature for 30 min prior to washing. Results are expressed as the mean percent of neutrophil adhesion ± standard deviation, where 100% was the total number of cells added per well. One represent experiment of three is shown.

3.2.2 THE EFFECT OF PURIFIED MRP-8 AND MRP-14 ON NEUTROPHIL ADHESION TO FIBRINOGEN

Due to the cross-reactivity of the monoclonal antibody mAb 5.5 ((Edgeworth et al., 1991) and data not shown), it was unclear whether the MRP-8/14 previously found deposited on the endothelium (Hogg et al., 1989) was the MRP-8/14 heterodimer, individual MRP-8 or MRP-14 proteins, or a combination of all three protein species. In order to test the hypothesis that either individual MRP-8 or MRP-14 may be involved in leukocyte adhesion, attempts were made to separate the MRP-8 and MRP-14 from the MRP-8/14 complex by affinity purification. Using reducing conditions, specific mAbs CF145 and CF557 (Hayward et al., 1986) were used to isolate MRP-8 and MRP-14, respectively. Despite the mAbs reported monospecificities, cross-contamination was always evident (data not shown). In addition, C_{18}-reverse phase HPLC methods were applied (Edgeworth et al., 1991) and although this method was more successful at separating MRP-8 and MRP-14 (data not shown), the yields of pure proteins were too low for the proteins to be tested in adhesion assays (Matthew Robinson, personal communication).

Instead, recombinant DNA constructs of the proteins, made in the laboratory by Dr. Paul Hessian, were used as a source of large quantities of purified MRP-8 and MRP-14 proteins. A single MRP-8 and two MRP-14 constructs, corresponding to the *in vivo* forms of the proteins, were purified using methods optimised by the ICRF Protein Isolation and Cloning Laboratory (Arnold Coffer, personal communication) and described in the Materials and Methods. The full-length recombinant MRP-14 protein, under non-reducing conditions, migrated on SDS-PAGE as a dimer at 28 kDa (**Figure 3.3A,a**); whereas the alternatively transcribed isoform, missing the N-terminal first four amino acids, including the only cysteine, was monomeric and migrated under the non-reducing conditions at ~14 kDa (**Figure 3.3A,b**). Under reducing conditions, full length recombinant MRP-14 migrated as expected at ~14kDa (**Figure 3.3B**). Recombinant MRP-8 protein, under non-reducing conditions, migrated as 3 bands at ~8, 16, and 24 kDa, suggesting it existed as a mixture of monomer, dimer and trimer (**Figure 3.3C, lane a**). For some experiments, monomeric MRP-8 was required and a reduced and alkylated monomeric form was used (**Figure 3.3C, lane c**).

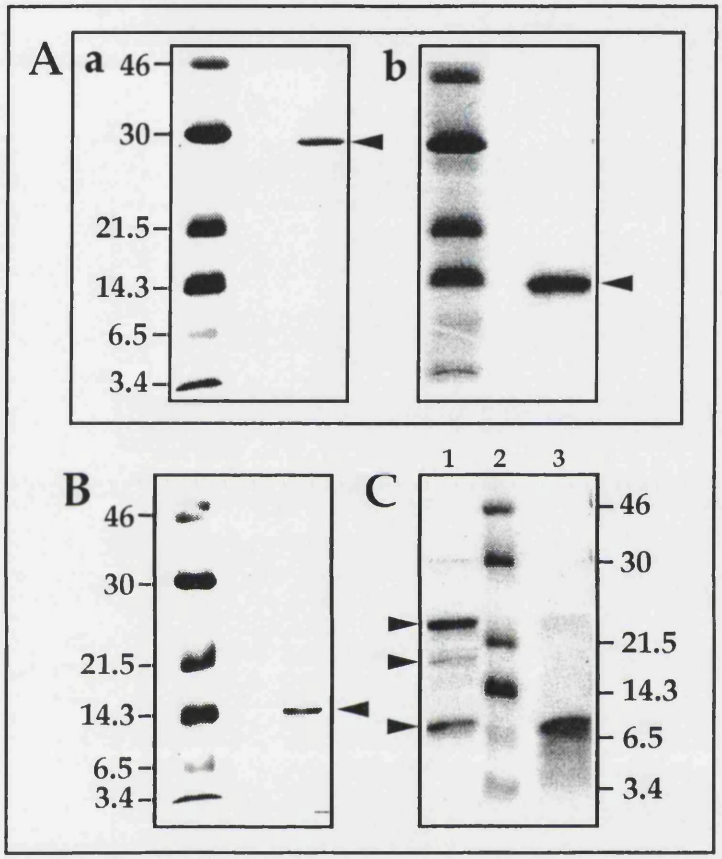

Figure 3.3: SDS-PAGE analysis of recombinant MRP-8 and MRP-14. 15% SDS-PAGE of **A.** Recombinant **a)** full-length and **b)** truncated MRP-14 under non-reducing and **B.** Recombinant full-length MRP-14 under reducing conditions. **C. Lane 1**, recombinant MRP-8 under non-reducing conditions; **lane 2** low molecular weight markers; **lane 3** recombinant MRP-8 after reduction and alkylation, under non-reducing conditions.

The full-length MRP-8 and MRP-14 recombinant proteins were first tested for their ability to induce neutrophil adhesion to fibrinogen. Dimeric MRP-14, at concentrations greater than 25μg/ml (~0.95μM), induced neutrophil adhesion to fibrinogen. MRP-8 tested over the same concentration range failed to induce any adhesion (**Figure 3.4**). The level of adhesion induced by MRP-14 at 100μg/ml (~3.8μM) was approximately 40% of that induced by 0.1μM fMLP (**Figure 3.4**).

Several S100 proteins, including MRP-14, have been shown to bind Zn^{2+} and in so doing, increase their affinity for Ca^{2+} presumably by a conformational change (Baudier et al., 1986; Raftery et al., 1996). The results in **Figure 3.5A** can be similarly interpreted, as the addition of 10μM Zn^{2+} increased the potency of MRP-14 to induce neutrophil adhesion by approximately 10 fold with maximum adhesion occurring at 1μM (≈25μg/ml dimeric) MRP-14 and equivalent to that induced by 0.1μM fMLP (**Figure 3.5A**). The response to 0.1μM fMLP was not similarly enhanced by 10μM Zn^{2+}; 100±2 % in the absence of Zn^{2+} compared to 120±14 % with Zn^{2+}. Titration of Zn^{2+} from 0-10 μM showed that MRP-14 function was optimal in physiological concentrations of 12-16 μM Zn^{2+} (Wellinghausen et al., 1997) (**Figure 3.5B**). Experiments to define specific Mg^{2+} and Ca^{2+} dependence were complicated by the fact that both MRP-8/14 and integrins can bind divalent cations; however, as expected, all binding was inhibited by the presence of EDTA (data not shown). A time course for neutrophil adhesion revealed that MRP-14- and fMLP-stimulated adhesion in the presence of 10μM Zn^{2+} with similar kinetics, with maximum binding occurring after 30 minutes at room temperature for both stimuli (**Figure 3.6**).

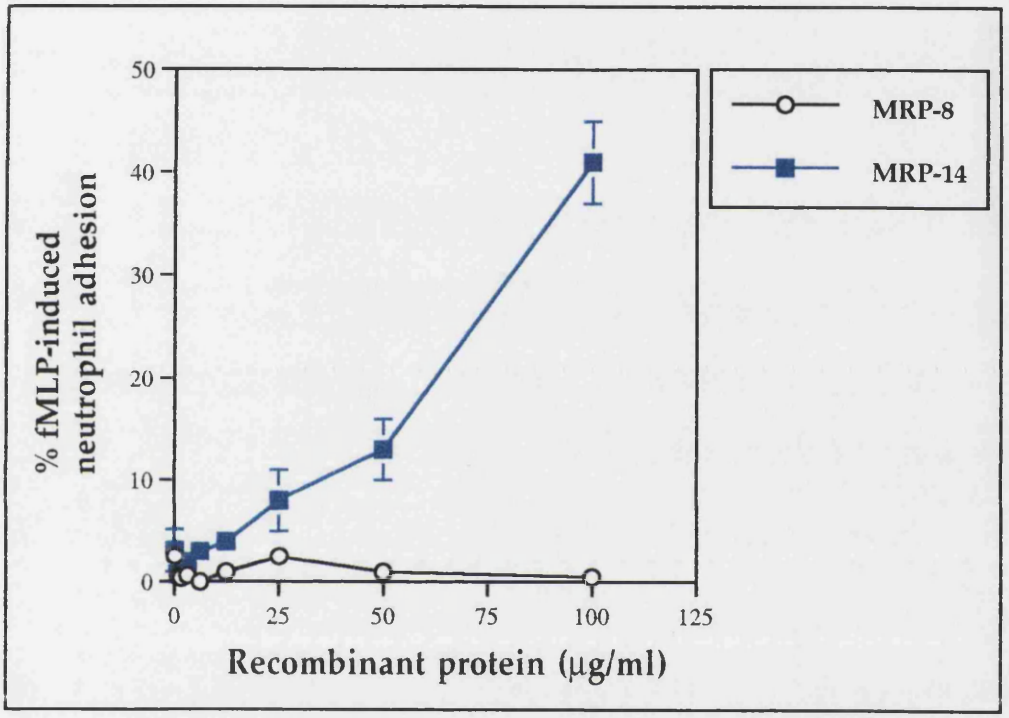

Figure 3.4: Recombinant MRP-14, but not MRP-8 induces neutrophil adhesion to fibrinogen. Dimeric recombinant full-length MRP-14 (—■—) and recombinant MRP-8 (—O—) were titrated from 0-100 µg/ml for their ability to induce neutrophil adhesion to fibrinogen. MRP-14, but not MRP-8, was able to induce adhesion to fibrinogen. Results are expressed as the mean % of neutrophils adherent in response to the positive control fMLP ± standard deviation. One experiment representative of six is shown.

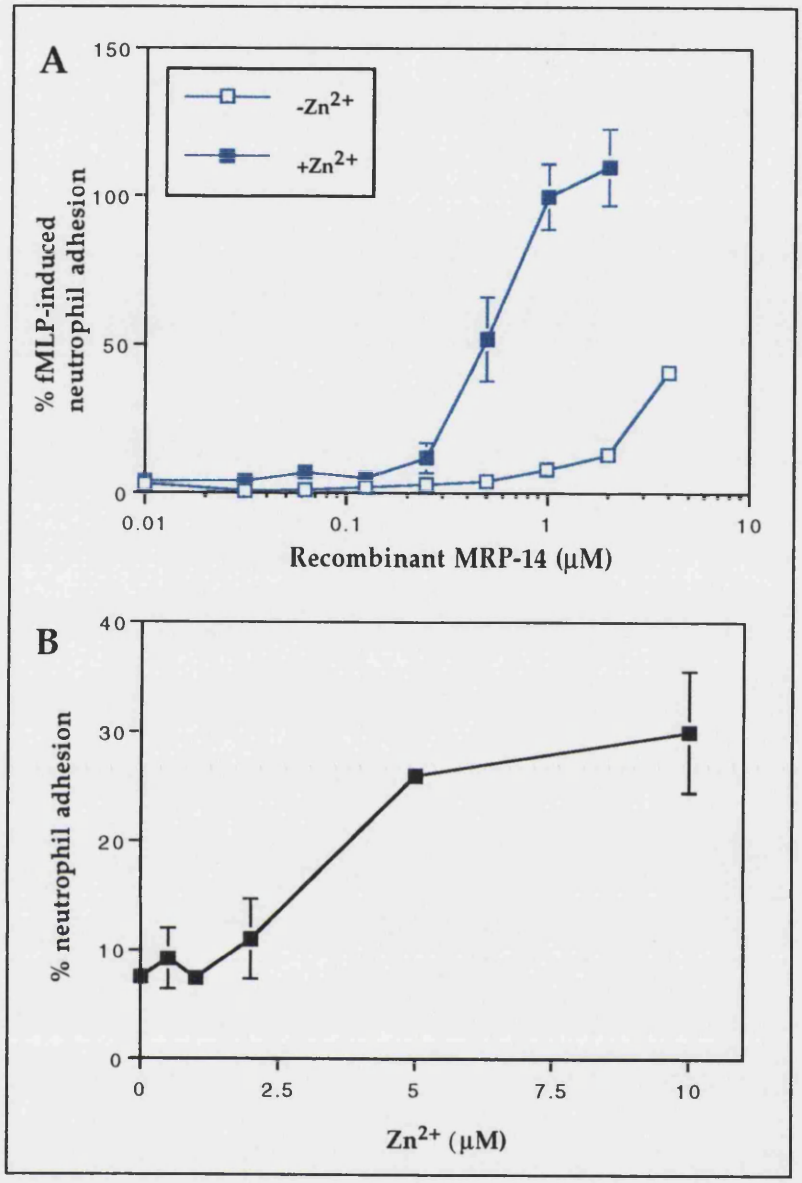

Figure 3.5: Optimal induction of neutrophil adhesion by recombinant MRP-14 is dependent on physiological concentrations of Zn^{2+}. A. Titration of MRP-14 from 0-4 μM in the presence (—■—) or absence (—□—) of 10μM Zn^{2+} measuring neutrophil adhesion to fibrinogen. Results are expressed as mean % fMLP-induced adhesion to fibrinogen ± standard deviation. One representative experiment of three is shown. **B.** Titration of Zn^{2+} from 0-10 μM to measure the effect on neutrophil adhesion to fibrinogen induced by 1 μM MRP-14. Results are expressed as mean % neutrophil adhesion ± standard deviation. One experiment representative of three is shown.

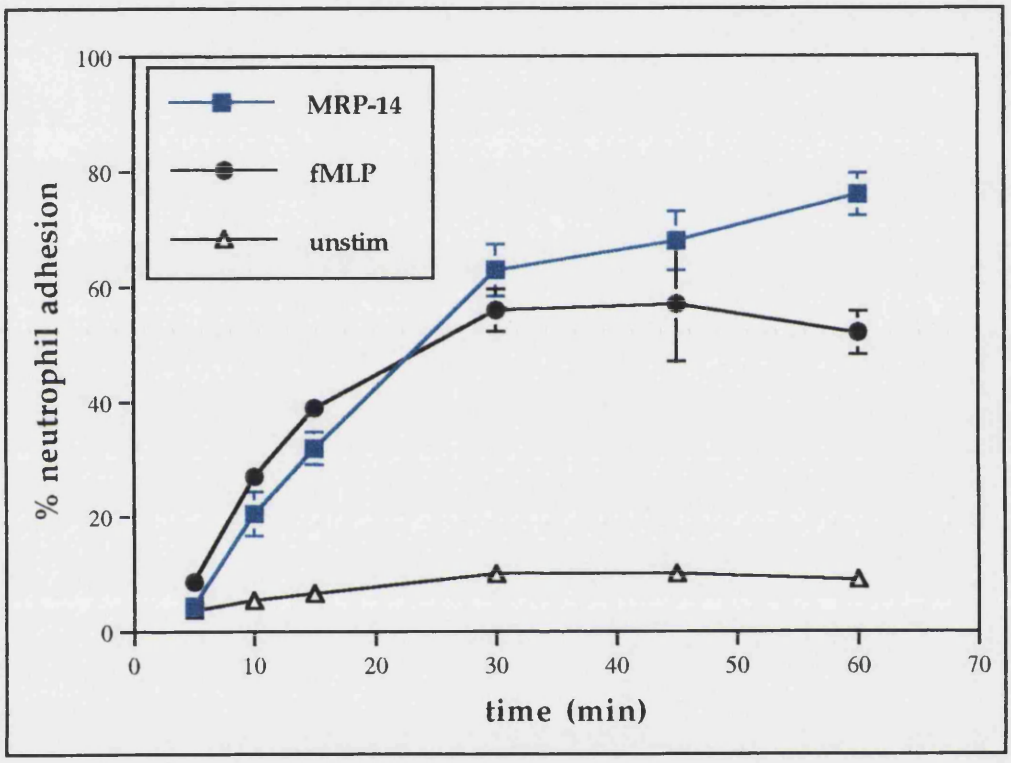

Figure 3.6: Time course of neutrophil adhesion to fibrinogen. Neutrophil adhesion to fibrinogen was measured from 0-60 min in the presence of buffer alone (—△—), 1μM MRP-14 (—■—) or 0.1μM fMLP (—●—). Results are presented as the mean % of adherent neutrophils ± standard deviation. One representative experiment of three is shown.

3.2.3 MRP-14, BUT NOT OTHER S100 PROTEINS, STIMULATES NEUTROPHIL ADHESION TO FIBRINOGEN

The ability to stimulate neutrophil adhesion was unique to MRP-14, as other recombinant S100 proteins tested, including MRP-8, S100A and S100B, failed to induce any adhesion, even in the presence of Zn^{2+} (**Figure 3.7A**). Furthermore, in the presence of Zn^{2+}, the native MRP-8/14 complex (**Figure 3.1B**) was still unable to induce any adhesion (**Figure 3.7A**). MRP-14-induced adhesion, like fMLP, was completely inhibited by the CD11b mAb LPM19c confirming that adhesion was mediated by the β_2 integrin Mac-1 (CD11b/CD18) (**Fig. 3.7B**).

The previous experiments described the ability of recombinant dimeric MRP-14 to stimulate adhesion. A comparison was therefore made between the dimeric and monomeric recombinant proteins to determine if dimerisation was required for MRP-14 function. Titration from 0-2 μM showed that the two proteins were identical in both their efficacy and potency, inducing maximum adhesion of 40% neutrophils at 1μM MRP-14 (**Figure 3.8**). These results indicate that the monomer was the functional unit.

There is increasing evidence that the presence of bacterial LPS (endotoxin) can affect neutrophil function. LPS alone is insufficient to induce neutrophil adhesion (Hailman et al., 1996); however, it is known to prime neutrophils (Doerfler et al., 1994; Surette et al., 1993). Every effort was made, therefore, to minimise endotoxin contamination in all assays. Despite this, the recombinant proteins produced were unavoidably contaminated with endotoxin. To eliminate the possibility that endotoxin in the recombinant MRP-14 protein solution was responsible for function, endotoxin removal was performed (see Materials and Methods) and the "endotoxin free" sample was tested in the neutrophil adhesion assay. The levels of endotoxin in the original monomeric MRP-14 sample, as measured by the *Limulus* amoebocyte lysate assay, was ≈ 60 ng/ml; whereas the levels of the "endotoxin free" sample were ≤ 3 pg/ml. Such low levels in the "endotoxin free" sample were acceptable considering the

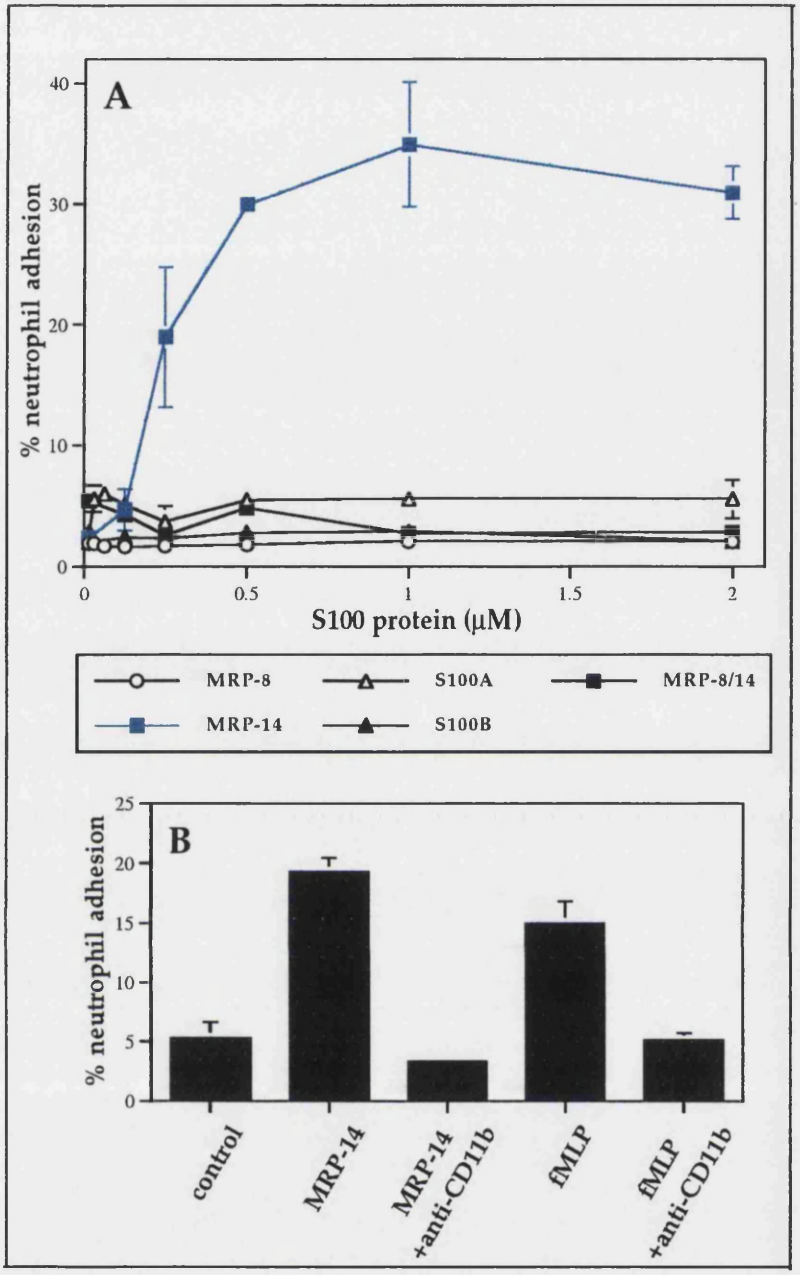

Figure 3.7: MRP-14, but not other recombinant S100 family members or native MRP-8/14, can induce neutrophil adhesion to fibrinogen. A. Recombinant MRP-14 (—■—), MRP-8 (—○—), S100A (—△—), S100B (—▲—) and native human MRP-8/14 (—■—) were titrated from 0-2 μM for their ability to induce neutrophil adhesion to fibrinogen. **B.** The adhesion induced by MRP-14 (1μM), like fMLP (0.1μM), was Mac-1 (CD11b) dependent as adhesion was inhibited by CD11b mAb 2LPM19c at 20 μg/ml. Results are expressed as the mean % neutrophil adhesion ± standard deviation. The data shown are from one experiment representative of six.

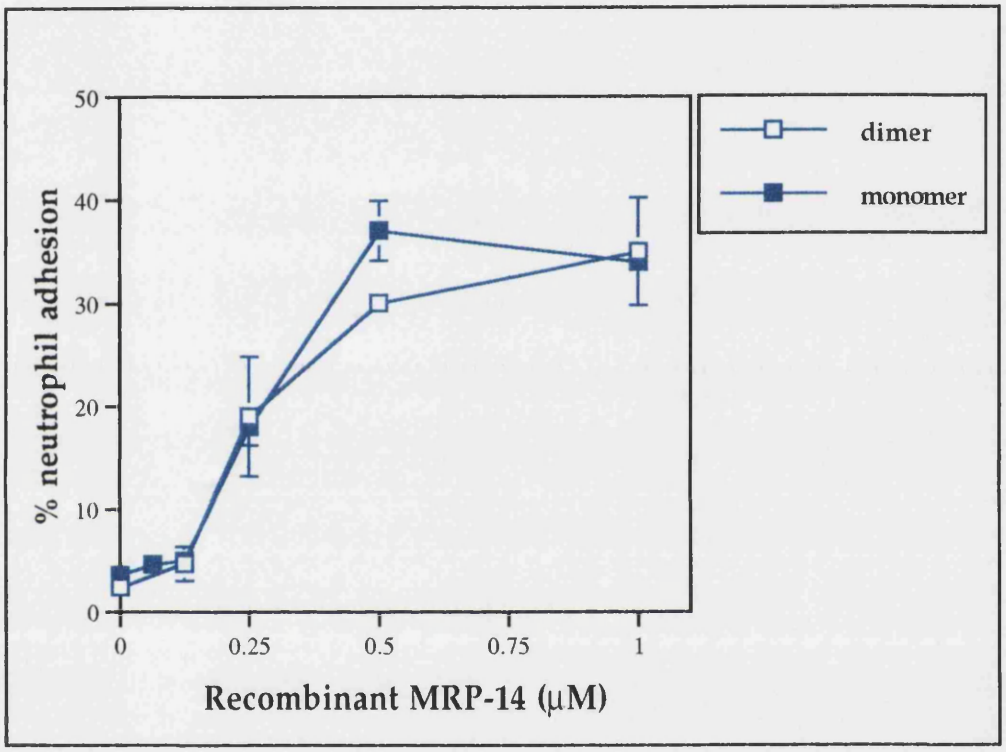

Figure 3.8: Comparison of monomeric and dimeric recombinant MRP-14 for its ability to induce neutrophil adhesion. Monomeric (—■—) and dimeric (—□—) recombinant MRP-14 were titrated from 0-1 μM for their ability to induce neutrophil adhesion to fibrinogen. Results are expressed as the mean % neutrophil adhesion ± standard deviation. The data shown are from one experiment representative of three.

protein was diluted at least 200 fold in each experiment. Endotoxin removal did not alter the protein concentration of the sample (data not shown) and the "endotoxin free" sample was as efficacious as the sample with endotoxin titrated in Figure 3.8 and may be even more potent with maximum adhesion induced at 0.25μM (**Figure 3.9A**). In addition, LPS alone was titrated into the adhesion assay buffer from 0-1μg/ml. This titration incorporated the levels of endotoxin present in the dilutions of the endotoxin-contaminated MRP-14 used. No adhesion above background was induced with any concentration tested (**Figure 3.9B**). Thus, the endotoxin in the protein sample was not contributing to the adhesion induced by MRP-14, either directly or by acting as a cofactor.

3.2.4 THE EFFECT OF MRP-8 ON MRP-14 FUNCTION

Experiments to assess the effect of MRP-8 on MRP-14-induced adhesion were prompted by the inability of the native MRP-8/14 heterodimer to induce neutrophil adhesion (**Figure 3.7A**). Titration of MRP-8 from 0.125 to 10μM caused a dose dependent inhibition of 2μM MRP-14-induced adhesion (**Figure 3.10**), with 50% inhibition occurring at an average ratio of 1:1 MRP-14 to MRP-8. MRP-8, over the same concentration range, had no effect on 0.1μM FMLP-induced adhesion (**Figure 3.10**). To eliminate the possibility that MRP-8 inhibition occurred by Ca^{2+} depletion, another S100 protein, S100A, was similarly titrated and had no inhibitory effect on MRP-14-induced adhesion (**Figure 3.10**). The inhibition of MRP-14 function by MRP-8 was concomitant with the expression of the MRP-8/14 complex specific epitope, recognised by mAb 27E10 (Bhardwaj et al., 1992) (**Figure 3.11**). MAb 27E10 did not react with monomers of isolated MRP-8 or MRP-14, but reacted in a dose-dependent manner only with 1μM MRP-14 co-incubated with increasing concentrations of MRP-8 up to 8μM (**Figure 3.11**). The ability of MRP-8 to inhibit MRP-14-induced adhesion and the coincident heterodimer formation suggests that the mechanism of MRP-8 inhibition is by direct binding to MRP-14 with a 1:1 stoichiometry between the two proteins. This identifies MRP-8 as a physiological antagonist of MRP-14.

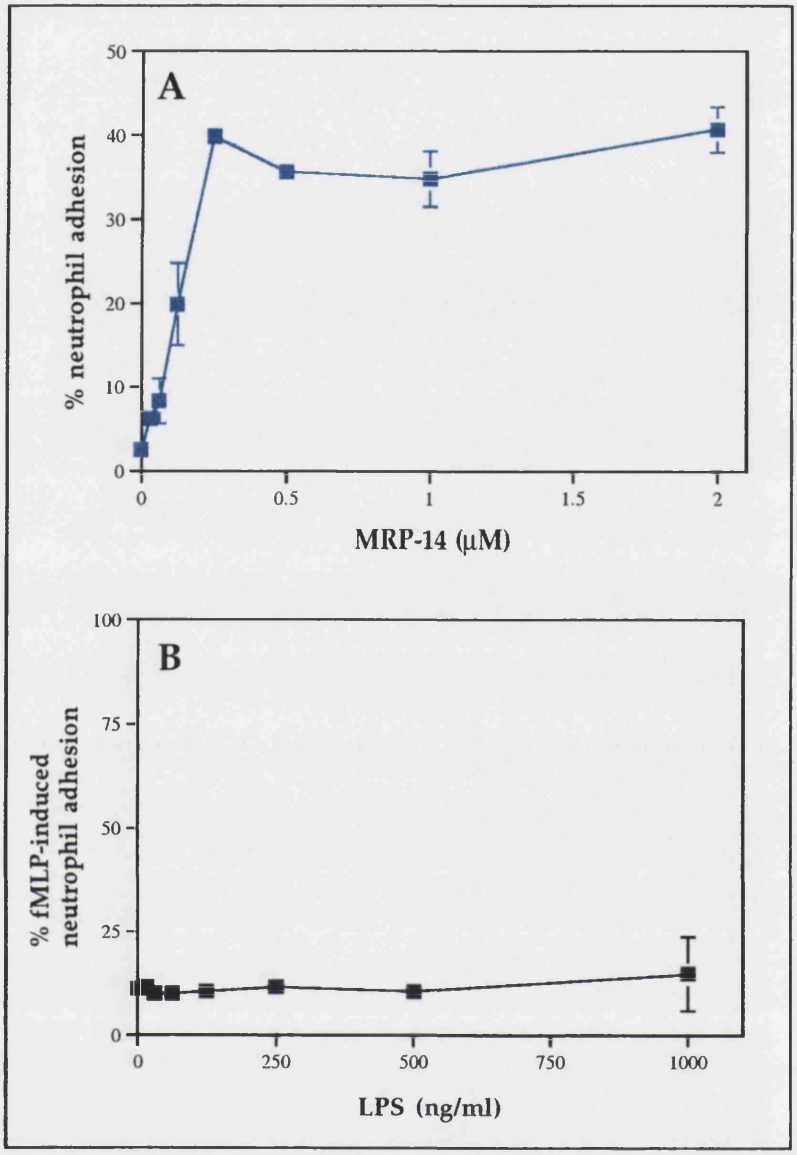

Figure 3.9: Lipopolysaccharide does not participate in MRP-14 stimulation of neutrophil adhesion. A. Monomeric MRP-14 at 400μM containing ≤ 3 pg/ml of endotoxin was titrated from 0-2 μM for the ability to induce neutrophil adhesion to fibrinogen. Results are expressed as the mean % of neutrophil adhesion ± standard deviation. The data shown are from a single experiment. **B.** Lipopolysaccharide (LPS) was titrated from 0-1000 ng/ml for the ability to stimulate neutrophil adhesion to fibrinogen. Results are expressed as the mean % of fMLP-stimulated neutrophil adhesion ± standard deviation. The data shown are from one experiment representative of three.

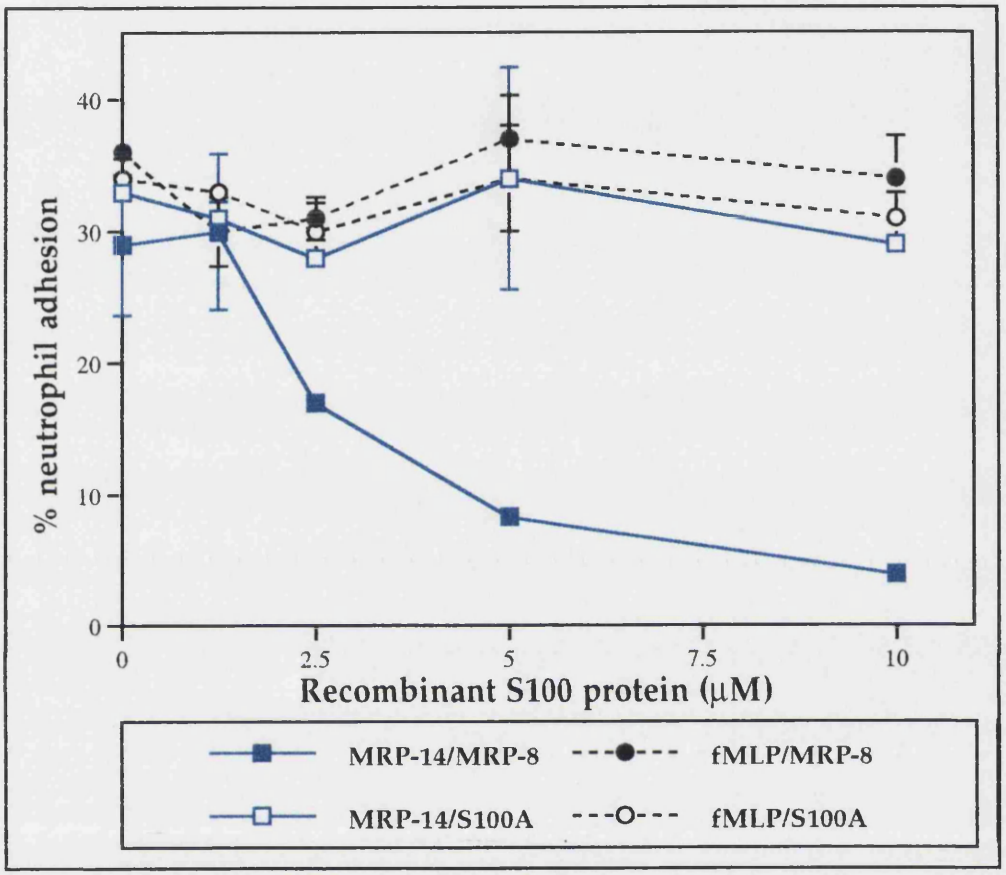

Figure 3.10: The effect of MRP-8 on MRP-14-induced adhesion. Recombinant monomeric MRP-8, titrated from 0-10 μM, inhibits the adhesion to fibrinogen induced by 1μM recombinant MRP-14 (—■—) but not that induced by 0.1μM fMLP (- - -●- -). Control S100A does not inhibit MRP-14 (—□—) nor fMLP-induced (—○—) adhesion. The data shown are from one experiment representative of six and are expressed as mean % neutrophil adhesion ± standard deviation.

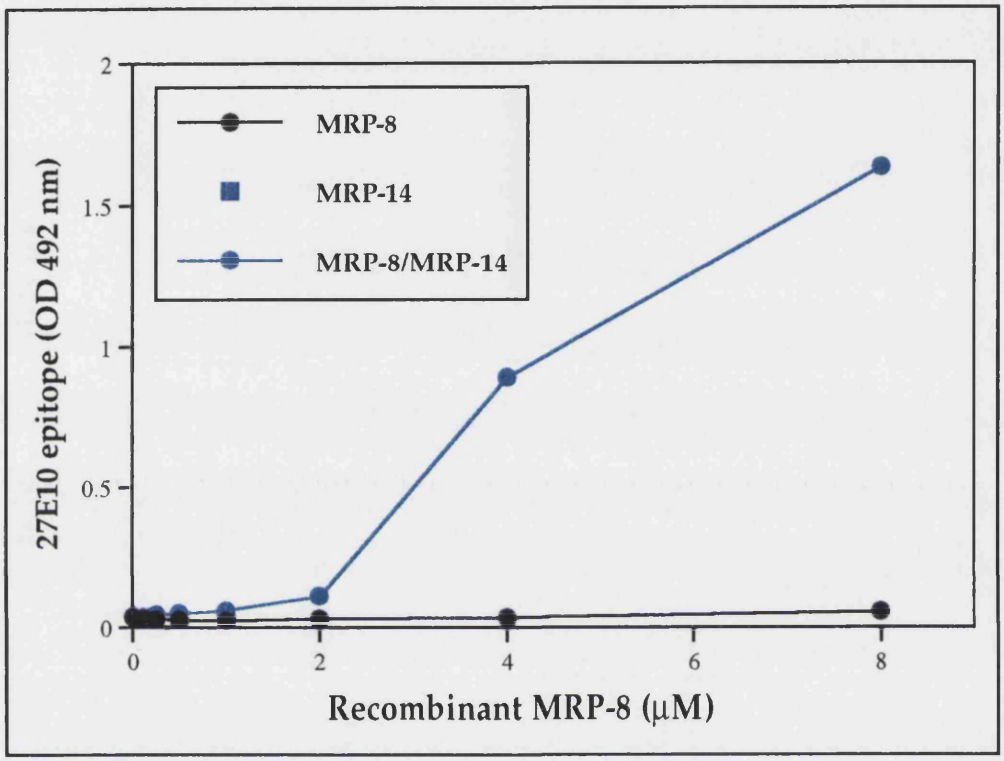

Figure 3.11: The formation of MRP-8/14 heterodimer from recombinant MRP-8 and MRP-14. Titration of monomeric MRP-8 from 0-8 μM in the presence (—●—) of 1 μM MRP-14, but not in the absence (—●—) results in the expression of a complex specific epitope recognised by mAb 27E10. MRP-14 alone (1 μM) does not react with mAb 27E10 (■). MAb 27E10 expression is represented optical density (OD) at 492 nm. The data shown are from one experiment representative of three.

3.2.5 THE EFFECT OF MRP-14 ON β_2 INTEGRIN LFA-1 ADHESION TO ICAM-1

From the results generated so far, MRP-14 has been identified as an activator of neutrophil Mac-1 adhesion. It remained to be tested whether MRP-14 affected other neutrophil β_2 integrins, such as LFA-1. MRP-14 did induce adhesion to immobilised ICAM-1Fc, with maximum binding of ~50% occurring at 1μM MRP-14 (**Figure 3.12A**). The binding induced by MRP-14 to ICAM-1 was fully inhibited by a blocking Mac-1 mAb (2LPM19c) and no blocking was seen with the anti-LFA-1 mAb (MEM25). No further blocking was seen when the anti-LFA-1 and Mac-1 mAbs were used in combination (**Figure 3.12B**). This data supports the fact that adhesion to ICAM-1 induced by MRP-14 was solely mediated by Mac-1. In contrast, some adhesion induced by fMLP was mediated by LFA-1 (~30%), although the majority was still Mac-1-dependent (~70%). Completely inhibition of fMLP-stimulated adhesion to ICAM-1 was only achieved with a combination of anti-LFA-1 and Mac-1 mAbs (**Figure 3.12B**).

3.2.6 MRP-14 IS A MODULATOR OF MAC-1 AFFINITY

An alternative method of activating integrin-mediated adhesion is by a direct conformational change in the integrin itself, resulting in an increase in affinity for ligand (reviewed in (Stewart and Hogg, 1996)). This increased integrin affinity is measured by the ability to bind ligand in solution. MRP-14 induces neutrophil binding of soluble FITC-fibrinogen with saturation at 10nM (**Figure 3.13**). FMLP also stimulates soluble fibrinogen binding, although saturation occurs at a higher fibrinogen concentration (20nM) (**Figure 3.13**). Despite differences in saturating concentrations, the maximum amount of soluble fibrinogen binding, however, is the same for both stimuli (equivalent maximum fluorescence values) (**Figure 3.13**). As predicted from the immobilised fibrinogen assays, MRP-8/14 complex did not stimulate any soluble FITC-fibrinogen binding (data not shown). The specificity of binding is shown by the ability of 10 fold excess unlabelled fibrinogen to reduce FITC-fibrinogen binding to background levels (**Figure 3.14A**). Removal of divalent cations (**Figure 3.13**) and blocking with a CD11b mAb showed the soluble fibrinogen binding induced by MRP-14 to be Mac-1-dependent with no involvement of other β_2 integrins, such as p150,95 which also recognises fibrinogen (**Figure 3.14B**) (Loike et al., 1991). The background binding of FITC-fibrinogen to untreated neutrophils (**Figure 3.13**) was also attributed to Mac-1 (data not shown).

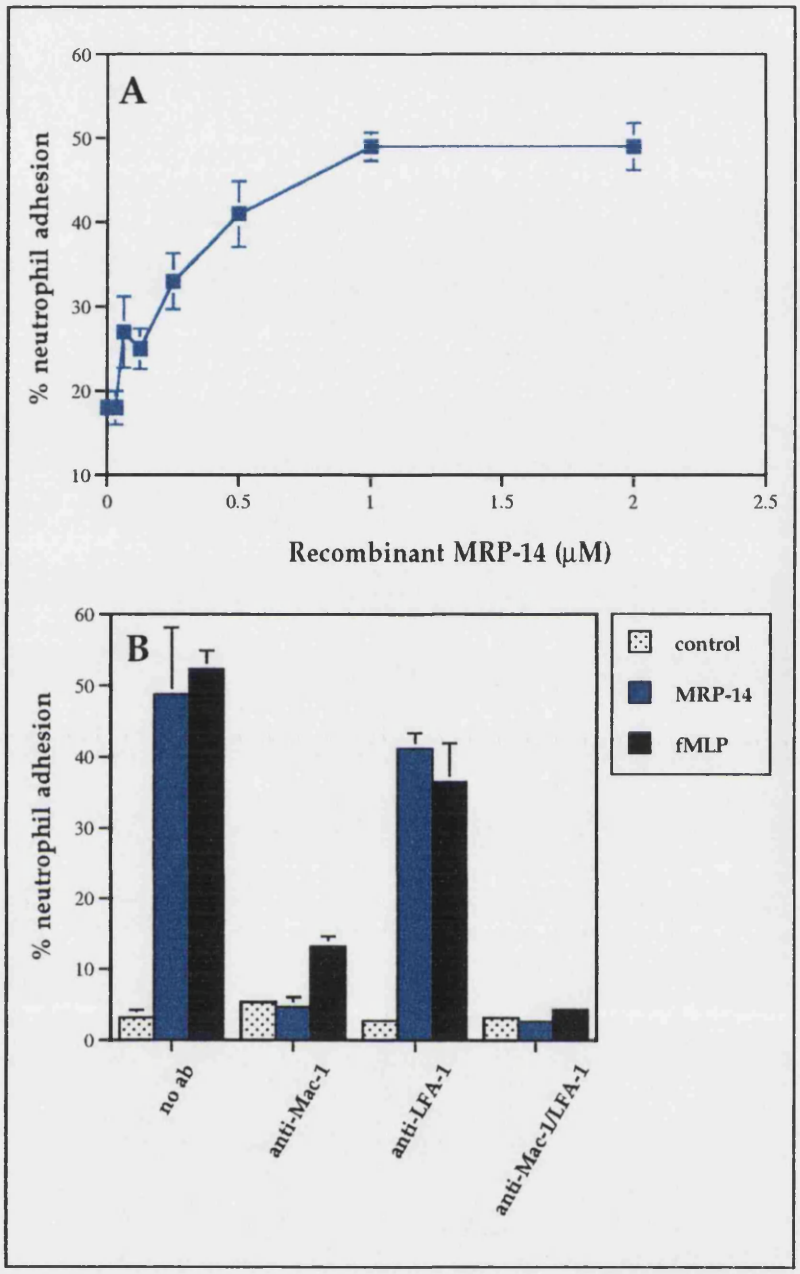

Figure 3.12: The effect of MRP-14 on neutrophil adhesion to ICAM-1. **A.** Titration of recombinant MRP-14 from 0-2 μM for the ability to induce adhesion to ICAM-1Fc. **B.** Monoclonal antibody inhibition of adhesion induced in response to buffer alone, 1μM MRP-14, or 0.1μM fMLP. Anti-Mac-1 (mAb 2LPM19c) and anti-LFA-1 (mAb MEM25) mAbs, both IgG1, were used at 20 μg/ml. Results are expressed as the mean % neutrophil adhesion ± standard deviation. The data shown are from one experiment representative of five.

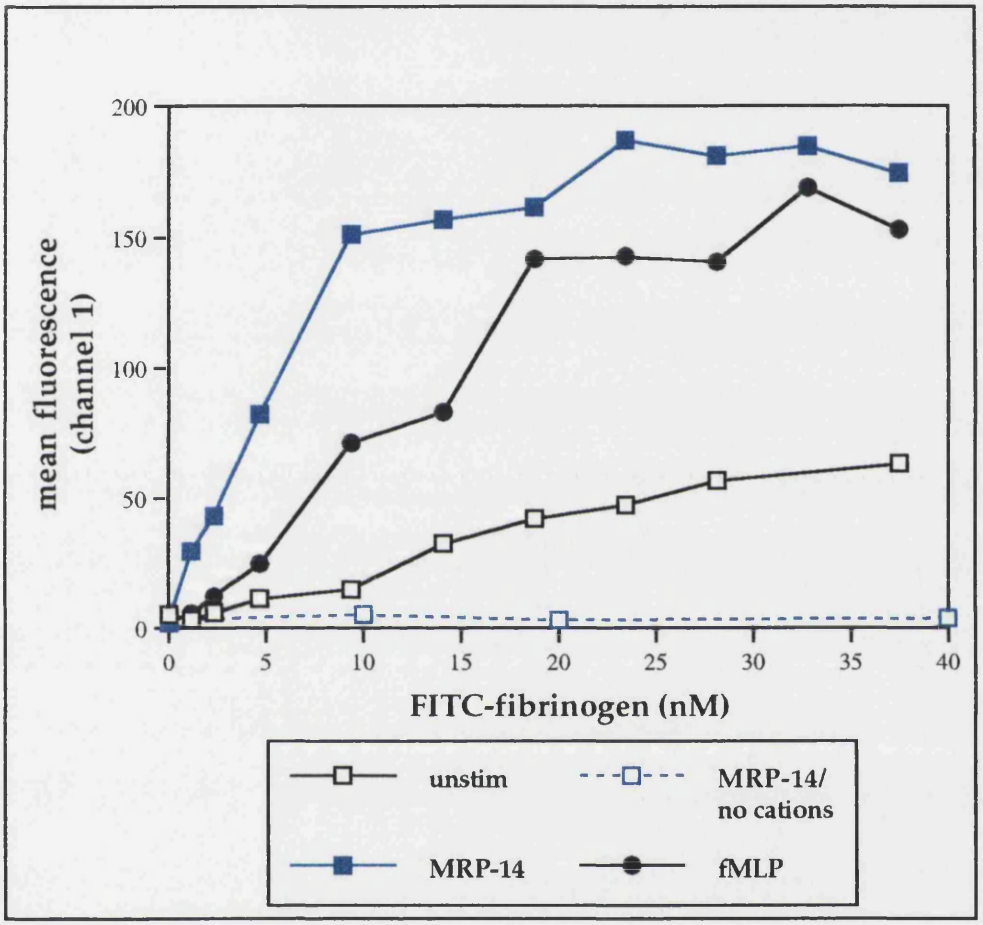

Figure 3.13: The effect of MRP-14 and fMLP on the binding of soluble fibrinogen to neutrophil Mac-1. MRP-14 (1μM) (—■—) and fMLP (0.1μM) (—●—) induce binding of FITC-fibrinogen to neutrophil (n=6). For MRP-14, binding is saturated at 10nM FITC-fibrinogen and is cation-dependent (- - -□- - -) (n=3). Unstimulated cell binding of FITC-fibrinogen is indicated (—□—) (n=6).

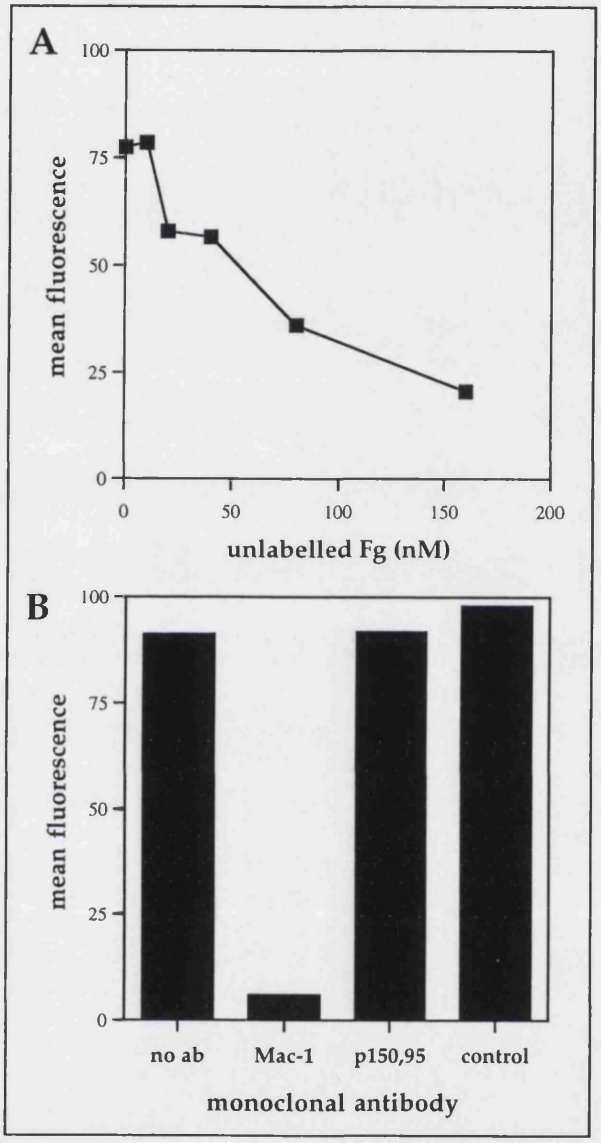

Figure 3.14: The blocking of MRP-14-stimulated FITC-fibrinogen binding. A. MRP-14-stimulated binding of 20nM FITC-fibrinogen to neutrophils is inhibited by unlabelled fibrinogen (n=3). **B.** FITC-fibrinogen (20nM) binding to MRP-14-stimulated neutrophils is inhibited by the CD11b blocking mAb 2LPM19c, but not by the CD11c blocking mAb 3.9, nor an IgG1 control mAb (52U) (n=3). MAbs were used at concentrations indicated in the Materials and Methods.

Another characteristic of high affinity integrins is the expression of "activation reporter" epitopes. To further assess the ability of MRP-14 to positively regulate β_2 integrin affinity, neutrophils were tested for the expression of an activation reporter epitope for β_2 integrins which is recognised by mAb 24 (Dransfield and Hogg, 1989; Stewart and Hogg, 1996). FMLP, at the concentration used for maximal adhesion (0.1μM), was able to induce increased mAb 24 epitope expression (**Figure 3.15A**). Similarly, MRP-14 was able to induce mAb 24 epitope expression on the surface of neutrophils (**Figure 3.15B**) in a concentration dependent manner which mirrored the induction of adhesion (data not shown). As expected from the immobilised fibrinogen assays, both soluble fibrinogen binding and mAb 24 expression in response to MRP-14 were inhibited by MRP-8 (data not shown). The mAb 24 expression stimulated by MRP-14 can be completely blocked by the Mac-1 blocking mAb 2LPM19c (**Figure 3.16**). The expression of this epitope further confirms that β_2 integrins are in a high affinity state as a result of exposure to MRP-14 (and fMLP). The results of Figure 3.16, together with those in Figures 3.12 and 3.14B, suggest that MRP-14 selectively activates Mac-1 and not LFA-1.

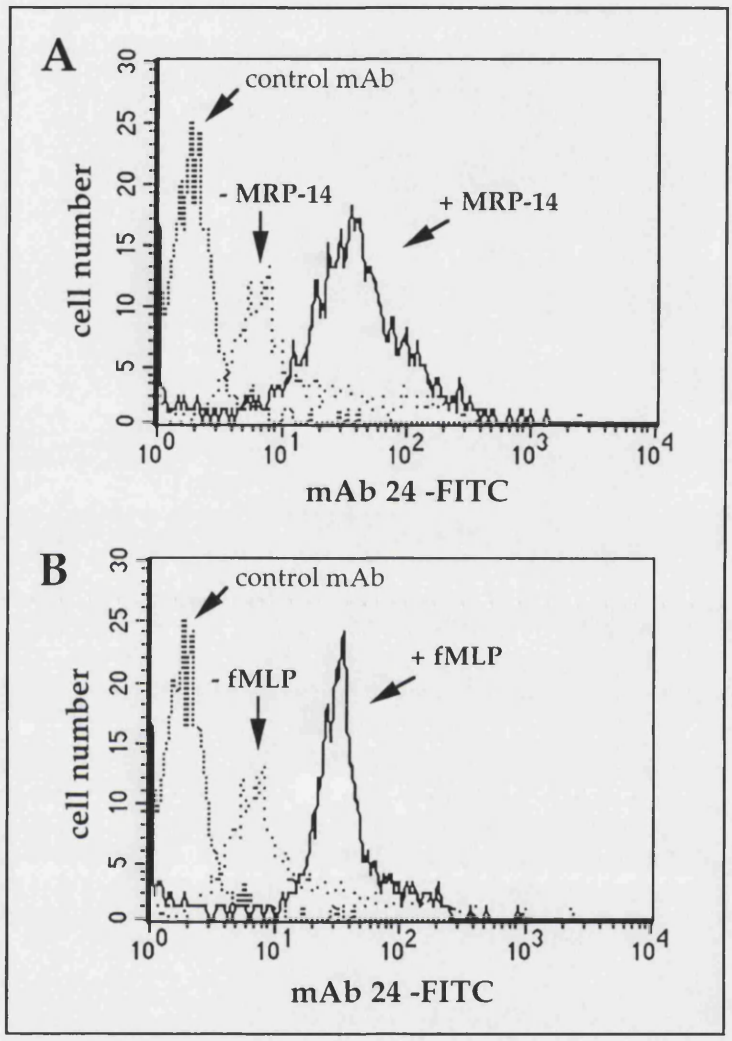

Figure 3.15: The activation of β_2 integrins on neutrophils by MRP-14. **A.** MRP-14 (1μM) and **B.** fMLP (0.1μM) activate the expression of a β_2 integrin activation epitope recognised by mAb 24. FITC-conjugated mAb 24 was used at 10μg/ml. Control FITC-conjugated mAb staining is indicated as (---------). Pretreatment levels of expression are indicated (·····) and post treatment levels as (———). The data shown are from one representative experiment of six.

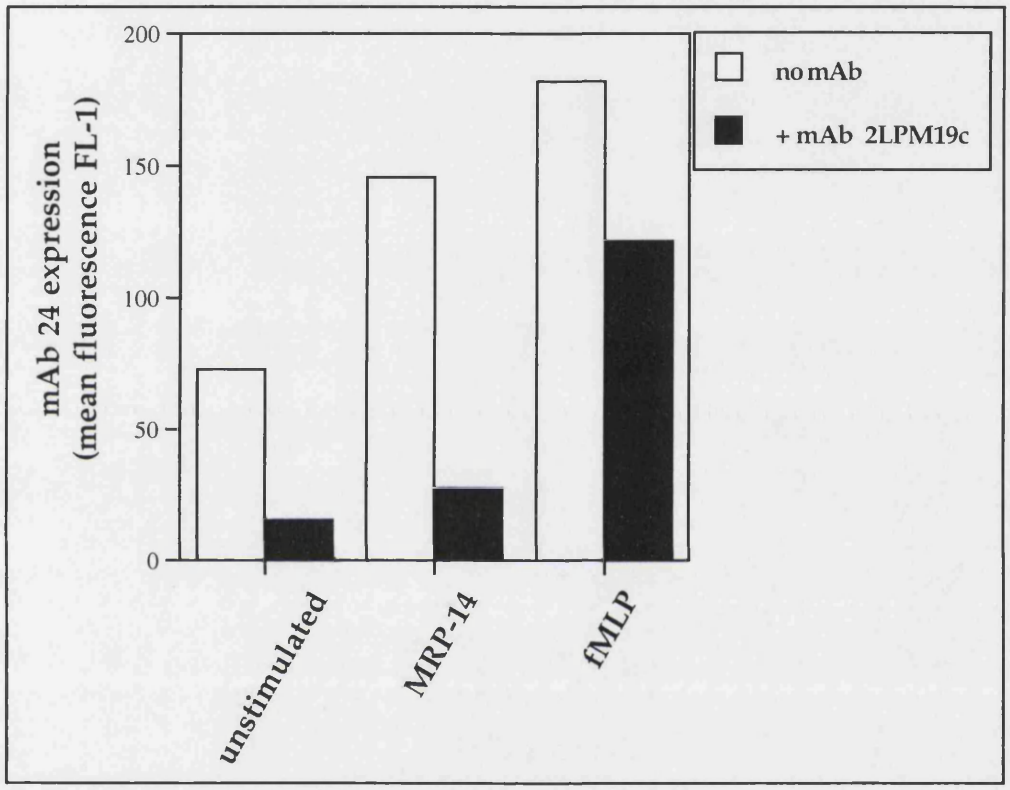

Figure 3.16: MAb 24 recognises activated Mac-1 on MRP-14-treated neutrophils. MAb 24 expression on neutrophils treated with 1μM MRP-14 is inhibited fully by the CD11b mAb 2LPM19c (10μg/ml). In contrast, fMLP-induced mAb 24 expression is only partially inhibited. Unstimulated mAb 24 expression is also inhibited by 2LPM19c. MAb 24 fluorescence levels without 2LPM19c are indicated as (□) and levels with 2LPM19c as (■). The data shown are from one representative experiment of three.

3.3 DISCUSSION

This chapter describes a modified method of purification of MRP-8/14 heterodimer from human neutrophils. In original experiments, blood from chronic granulocytic leukaemia (CGL) patients was used as a source of large numbers of neutrophils. These preparations contained obvious red blood cell (RBC) protein contamination. There was a concern that these contaminating proteins may have contributed to the inability of MRP-8/14 to affect neutrophil adhesion. To avoid the issue of contaminating proteins, cytosolic protein preparations from normal human neutrophils were used as a source of MRP-8/14. Although this required greater blood volumes and increased effort to isolate the neutrophils to greater purity ($\geq$ 98%), the resultant MRP-8/14 heterodimer contained almost no contamination even after one round of anion-exchange chromatography. This was advantageous as cation-exchange chromatography, in buffers of pH 4.5, resulted in some precipitation of MRP-8/14. The concerns with precipitation were twofold: firstly, the loss of MRP-8/14, and secondly, and more importantly, the possibility that the material was destabilised and no longer functional. This modified method thus provided a greater yield of purer and potentially more functional MRP-8/14 heterodimer.

Despite these efforts the MRP-8/14 heterodimer was still unable to affect either stimulated or unstimulated neutrophil adhesion. Instead, MRP-14 monomer was identified as an activator of neutrophil Mac-1 adhesion to fibrinogen. This adhesion was dependent on cations, in particular Zn^{2+}, where the potency of MRP-14 is increased 10 fold by the addition of physiological concentrations. MRP-14 is known to bind Zn^{2+} (Raftery et al., 1996) and there is a potential Zn^{2+} binding site in the C-terminus, represented by the amino acids ^{90}HEKMH^{94} (Baudier et al., 1986; Raftery et al., 1996). Based on studies with S100B (Baudier et al., 1986) it can be assumed for MRP-14 that the addition of Zn^{2+} causes conformational changes, although this remains to be tested. MRP-14 is unique among the S100 family members tested, including the Zn^{2+}-binding S100B, in its ability to stimulate neutrophil Mac-1 adhesion. MRP-8, the heterodimeric partner of MRP-14, does not induce adhesion either and instead has been identified as a natural and specific inhibitor of MRP-14 function. This inhibition occurs as a direct interaction in a 1:1 ratio of MRP-8 and MRP-14 and the subsequent heterodimer formation. This finding is supported by the fact that the native MRP-8/14 complex is unable to induce adhesion. It is the complex which exists intracellularly (Edgeworth et al., 1991) and is expressed on the myeloid cell surface (Bhardwaj et al., 1992) and secreted, at least by monocytes (Rammes et al., 1997). There are, however, some instances where MRP-14 is expressed alone in

acute inflammatory disorders (Delabie et al., 1990; Zwadlo et al., 1988). In addition, MRP-14 has been detected as a monomer in serum (Brüggen et al., 1988), and isolated MRP-14 has recently been shown to be secreted from monocytes (Lügering et al., 1997). Whether can be expressed and secreted by myeloid cells remains to be determined. Conditions leading to dissociation of MRP-14 from the heterodimer also need to be investigated.

It could be assumed that the recombinant protein is representative of native MRP-14 as it is not glycosylated and only appears to be post-translationally modified by cation binding and by phosphorylation of the penultimate amino acid (Thr 113) (Dorin et al., 1987; Edgeworth et al., 1991; Odink et al., 1987; Raftery et al., 1996). The secretion of MRP-8/14 from monocytes has been shown to be a PKC-dependent event (Rammes et al., 1997), distinct from the PKC-independent phosphorylation and translocation of MRP-8/14 to the cell surface (Guignard et al., 1996; Lemarchand et al., 1992; Roth et al., 1993; van den Bos et al., 1996). This suggests that secreted MRP-14 need not necessarily be phosphorylated and that the phosphorylated membrane-associated form of MRP-8/14 may have a distinct intracellular function. Thus the recombinant MRP-14 protein appears to be representative of native MRP-14. The functions of native MRP-8 and MRP-14 proteins, however, still need to compared to their recombinant counterparts.

The fact that dimeric and monomeric MRP-14 are equivalent in potency and efficacy illustrates that the monomer is the functional unit. This contrasts MRP-14 with another extracellular S100 protein, S100B, whose function is dependent on dimerisation (Selinfreund et al., 1991; Winningham-Major et al., 1989). It also implies that the N-terminal Met-Thr-Cys-Lys, absent from monomeric MRP-14, is not required for MRP-14 function. The only region implicated in MRP-14 function is the C-terminal 26 amino acid tail with NIF homology (Freemont et al., 1989; Watt et al., 1983). MRP-14 is the largest of the S100 protein family, with 114 amino acids, and it is this extended tail sequence (amino acids 89-114) which distinguishes it from other S100 proteins. This tail region of MRP-14 is also homologous to the "contact" (domain 5 of the light chain) domain within the procoagulant protein high molecular weight kininogen (HMWK). This domain within HMWK is highly positively charged and is responsible for HMWK binding to endothelium (Reddigari et al., 1993) and negatively charged surfaces (DeLa Cadena and Colman, 1992; Kunapuli et al., 1993). This same domain is also responsible for binding to neutrophils and displacing bound fibrinogen (Gustafson et al., 1989) by binding to Mac-1 (Wachtfogel et al., 1994). Results published from our laboratory (Hessian et al., 1995) showed that MRP-14 (and MRP-8/14) can similarly bind to negatively charged surfaces (i.e. kaolin) and is

competitively inhibited by both HMWK and the domain homologous to MRP-14. Whether MRP-14 can bind to endothelium has yet to be determined; however, it could be speculated that the MRP-14 is released by monocytes or neutrophils and is tethered by this charged tail sequence to the vessel wall. Localised here the MRP-14 could then stimulate adhesion of neutrophils to the endothelium. However, it is also this tail sequence that has been ascribed neutrophil immobilising activity. It is possible that the same region bound to the endothelium is also the active site for activating neutrophil adhesion. In this way MRP-14 localised could cause the accumulation of neutrophils at sites of extravasation to inflammatory sites. Experiments are presently being developed in the laboratory to investigate the ability of peptides derived from the MRP-14 protein sequence to directly induce neutrophil adhesion or to inhibit the adhesion induced by full-length MRP-14.

The results presented in this chapter confirm the hypothesis that MRP-8/14 does play a role in neutrophil adhesion. MRP-14 has been identified as a inducer of neutrophil Mac-1 adhesion with MRP-8 being able to negatively regulate MRP-14 function by the formation of the MRP-8/14 heterodimer. With this information, it would be worth reinvestigating the results seen in Figure 1.7. The positive staining seen on the section may not represent MRP-8/14 complex but, alternatively, MRP-14 alone. Using the new mAbs generated against recombinant MRP-14 it would be worth investigating this possibility and to find circumstances where MRP-14 alone may be secreted. This would further confirm an *in vivo* role for MRP-14 in neutrophil adhesion as implicated from the results presented in this chapter.

THE EFFECT OF MRP-14 ON NEUTROPHIL FUNCTION

4.1 INTRODUCTION

The rapid recruitment of neutrophils from the blood to sites of tissue injury requires a cascade of well characterised adhesion events mediated by the selectins and the leukocyte β_2 integrins (CD11/CD18) (Springer, 1994). The selectins mediate tethering and rolling of leukocytes and the β_2 integrins, Mac-1 and LFA-1, are responsible for firm adhesion to and transmigration through endothelium (Springer, 1994). The function of these β_2 integrins is regulated, however, and they can become active *in vivo* following signalling through other receptors ("inside-out" signalling). Three families of molecules have been described to activate neutrophil β_2 integrin adhesion *in vitro*, and have also been proposed as candidates for *in vivo* integrin activation. These are the selectin family of adhesion molecules; the classic chemoattractants; and the extracellular adenosine nucleotides, ADP and ATP.

The selectins were considered as candidates for activation of β_2 integrins *in vivo* as their interactions with ligand on endothelium and leukocytes directly precede integrin binding (Butcher, 1991). Indeed, antibody cross-linking of L-selectin on neutrophils has been reported to induce Mac-1 activation and ligand binding (Gopalan et al., 1997 1055; Simon et al., 1995). It has also been reported that the interaction of ligand GlyCAM-1 with L-selectin causes β_2 integrin activation on naïve T cells (Hwang et al., 1996). It has been suggested, therefore, that L-selectin itself can transduce a signal into the neutrophil upon ligation and in turn activate β_2 integrins. The ability of E- and P-selectin ligation to similarly activate β_2 integrins has been investigated; however, the results generated have been controversial. More recent experiments have shown E- and P- selectin interactions to be unable to induce β_2 integrin adhesion in the absence of a second signal such as endotoxin or platelet activating factor (PAF) (Gopalan et al., 1997; Lorant et al., 1991). Another candidate family of neutrophil β_2 integrin activators are the classic chemoattractants, such as formyl-methionyl-leucyl-phenylalanine (fMLP), PAF and the chemokines, particularly IL-8. Interaction of these chemoattractants with their heterotrimeric G protein-coupled receptors on the neutrophil membrane is able to stimulate β_2 integrin adhesion in *in vitro* assays

(Carveth et al., 1989; Detmers et al., 1989). The third candidate family of β_2 integrin activators are the adenosine nucleotides, ADP and ATP, which operate through P_2-purinergic receptors on the neutrophil membrane (Forsberg et al., 1987). ADP and ATP can be released from activated platelets and endothelium and have been reported to activate Mac-1-mediated adhesion of monocytes (Altieri and Edgington, 1988) and myeloid cell lines (Altieri et al., 1990), as well as neutrophils (Freyer et al., 1988).
In addition to activating adhesion, triggering by L-selectin, chemoattractants and nucleotides has also been shown to stimulate neutrophil calcium flux (Baggiolini et al., 1994; Cowen et al., 1989; Crockett-Torabi et al., 1995) and L-selectin and chemoattractants also generate neutrophil respiratory burst (Baggiolini et al., 1994; Crockett-Torabi et al., 1995). Nucleotides do not themselves generate a neutrophil respiratory burst but are able to prime cells for the response to chemoattractants (Gasmi et al., 1994). All three candidate families of molecules cause upregulation of Mac-1 expression on the neutrophil membrane (Akbar et al., 1997; Baggiolini et al., 1994; Freyer et al., 1988; Simon et al., 1995). From these results it is obvious that activators of neutrophil adhesion also have major effects on neutrophil effector functions. It still remains unclear however, which of these signalling molecules operate *in vivo* and whether stimulation of β_2 integrin adhesion, chemotaxis and other neutrophil effector functions are all initiated by the same mediators. In view of the fact that MRP-14 is found *in vivo* deposited on the endothelium and can stimulate neutrophil adhesion, the possibility that MRP-14 might also activate neutrophil effector functions was investigated.

4.2 RESULTS

4.2.1 THE EFFECT OF MRP-14 ON NEUTROPHIL ADHESION MOLECULE EXPRESSION

As described above, the stimulated neutrophil undergoes a number of cell surface and intracellular changes. For example, engagement of certain cell surface receptors can invoke quantitative changes in expression of at least two adhesion molecules (Kishimoto et al., 1989). Characteristically, for the classic chemoattractants, there is shedding of L-selectin and an upregulation of Mac-1 on the neutrophil surface. As expected, fMLP used at 0.1μM gave the characteristic decrease in cell surface L-selectin expression (**Figure 4.1A**); however, MRP-14 at 1μM (or at concentrations up to 8μM-data not shown) failed to alter the levels of neutrophil L-selectin (**Figure 4.1B**). It was then investigated whether MRP-14, like fMLP (**Figure 4.1C**) might

cause upregulation of Mac-1 from cytoplasmic granules. However, MRP-14 at 1μM (or at concentrations up to 8μM-data not shown) failed to alter the levels of neutrophil Mac-1 (**Figure 4.1D**). Furthermore, MRP-14 was unable to affect the changes in adhesion molecule expression invoked by fMLP (data not shown). Purification of neutrophils from whole blood has been shown to cause an upregulation of Mac-1 expression (Macey et al., 1995). To be certain that the ability of MRP-14 to cause an upregulation in Mac-1 was not being masked by an increase due to purification methods, Mac-1 expression was measured in whole blood in response to MRP-14. As for purified neutrophils, no increase in Mac-1 expression was observed for MRP-14 treatment (**Figure 4.2A**); whereas, the characteristic increase was detected for cells treated with fMLP (**Figure 4.2B**).

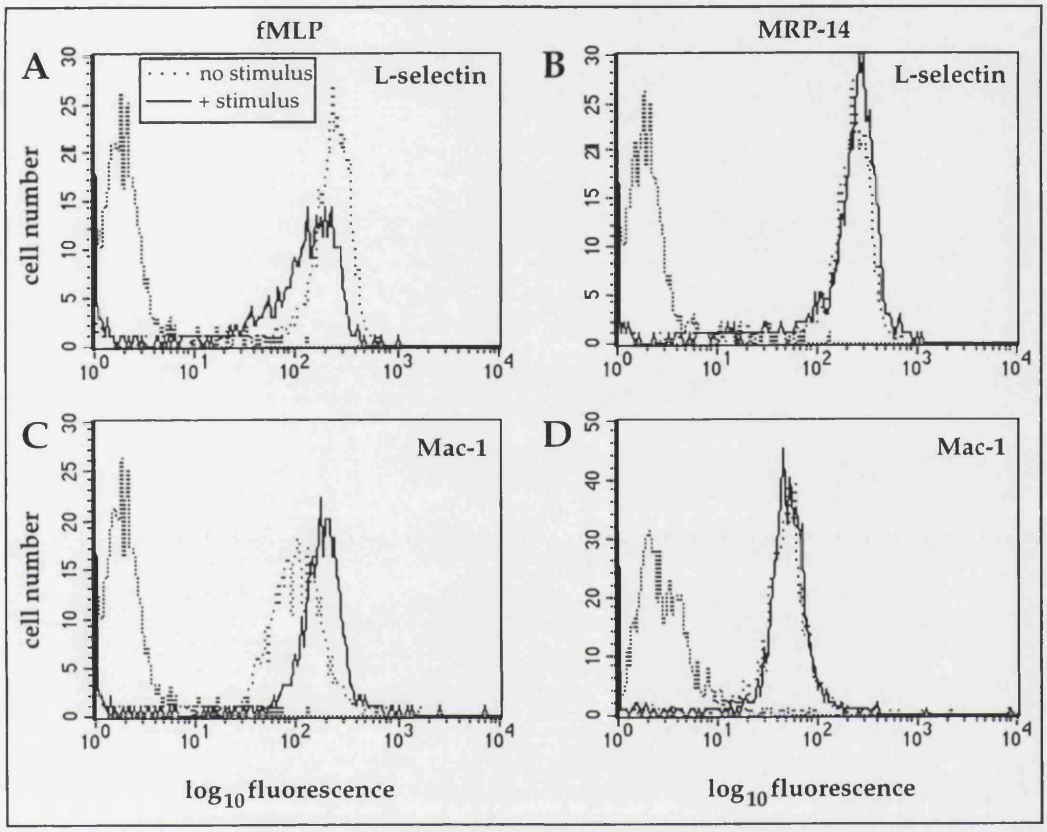

Figure 4.1: The effect of MRP-14 on neutrophil adhesion molecule expression. A. fMLP (0.1μM), but not **B.** MRP-14 (1μM), reduces expression of L-selectin using CD62L mAb LAM1.3. Likewise, **C.** fMLP, but not **D.** MRP-14, increases expression of the β_2 integrin Mac-1 using CD11b mAb ICRF44. MAbs were used at concentrations indicated in the Materials and Methods. Control Ab staining is indicated as (---------). Pretreatment levels of expression are indicated (·····) and post treatment levels as (———). The data shown are from one representative experiment of six.

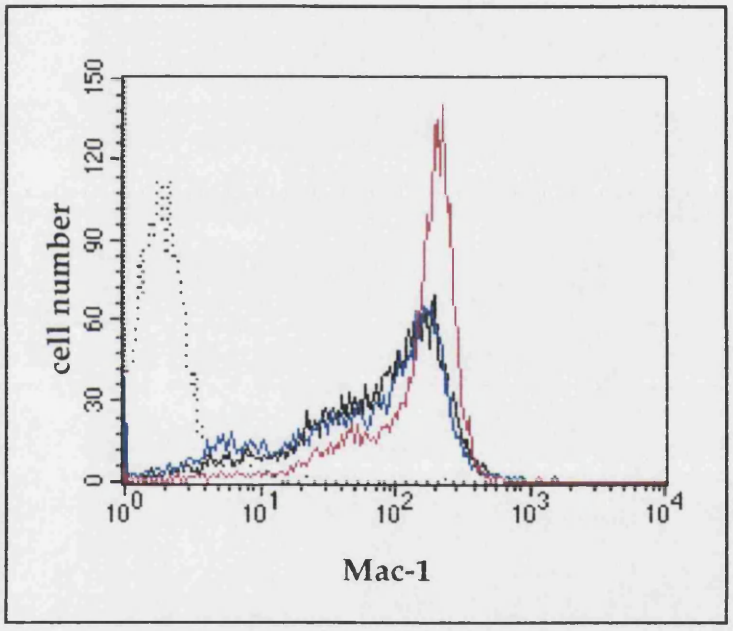

Figure 4.2: The effect of MRP-14 on Mac-1 expression on neutrophils in whole blood. A. fMLP (0.1μM) (———), but not **B.** MRP-14 (1μM) (———), increases expression of the β_2 integrin Mac-1 on neutrophils in whole blood as measured by FITC-conjugated ICRF44. Unstained cells are indicated as (·····) and unstimulated cell Mac-1 expression is indicated by (———). The data shown are from a single experiment.

4.2.2 THE EFFECT OF MRP-14 ON NEUTROPHIL EXOCYTOSIS, CALCIUM FLUX, AND SUPEROXIDE PRODUCTION

Neutrophil stimulants, such as classical chemoattractants, also cause exocytosis of granules additional to those containing Mac-1, with β-glucuronidase release from azurophilic granules frequently taken as a measure of this activity (see (Baggiolini et al., 1993)). FMLP (0.1μM) stimulated the release of β-glucuronidase from neutrophils as measured by the levels of phenolphthalein in the supernatant (**Figure 4.3**). MRP-14 (2μM), on the other hand, failed to induce release of β-glucuronidase (**Figure 4.3**), further supporting the inability of MRP-14 to induce neutrophil granule exocytosis. In addition to changes in adhesion molecule expression, activated neutrophils can increase intracellular Ca^{2+} $[Ca^{2+}]_i$. Initially, there is a release from intracellular stores, followed by an influx across the cell membrane. FMLP gave the characteristic response of intracellular Ca^{2+} flux, whereas 1μM MRP-14 failed to induce such a Ca^{2+} flux even after 30 min (**Figure 4.4** and data not shown). Concentrations up to 4μM MRP-14 still failed to induce a Ca^{2+} flux (data not shown). In addition, MRP-14 had no effect on the Ca^{2+} mobilisation induced by fMLP (data not shown). The possibility that MRP-14 might activate the neutrophil respiratory burst was also investigated (see (Baggiolini et al., 1993)). FMLP at 0.1μM (**Figure 4.5A**), PMA and the chemokine IL-8 (data not shown) caused neutrophil activation of the NADPH oxidase as detected by production of superoxide, but MRP-14 at 1μM (or concentrations up to 8μM-data not shown) failed to stimulate any production of superoxide (**Figure 4.5B**). In some experiments it appeared that MRP-14-treated neutrophils may have had a lower level NADPH oxidase activity than unstimulated cells, as determined by di-hydrorhodamine fluorescence (data not shown).

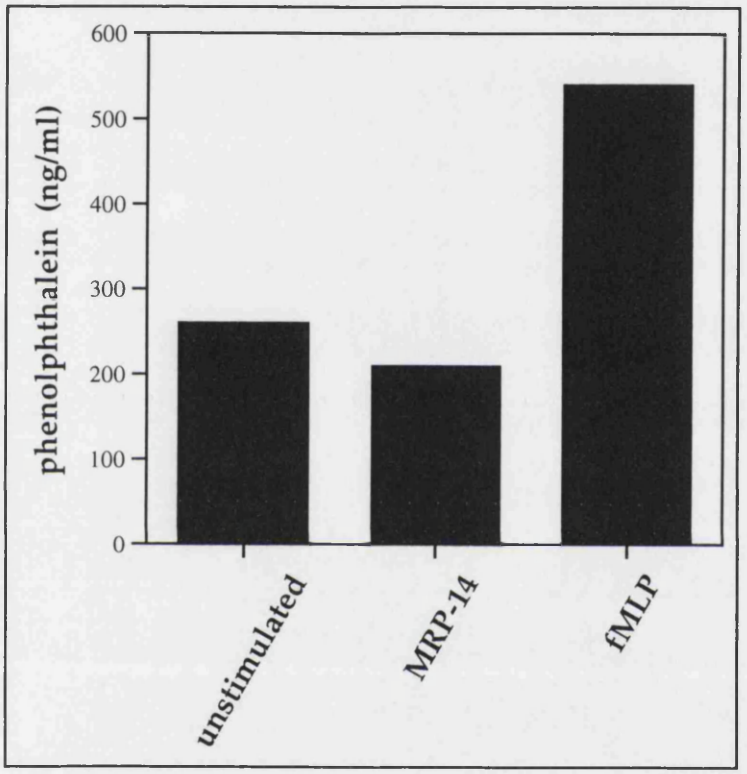

Figure 4.3: The effect of MRP-14 on β-glucuronidase release from neutrophils as a measure of exocytosis. Neutrophils were stimulated to release β-glucuronidase by fMLP (0.1μM) but not by MRP-14 (2μM). The measurement of phenolphthalein in supernatants was used as a correlate of β-glucuronidase release. Results are from a single experiment, representative of two.

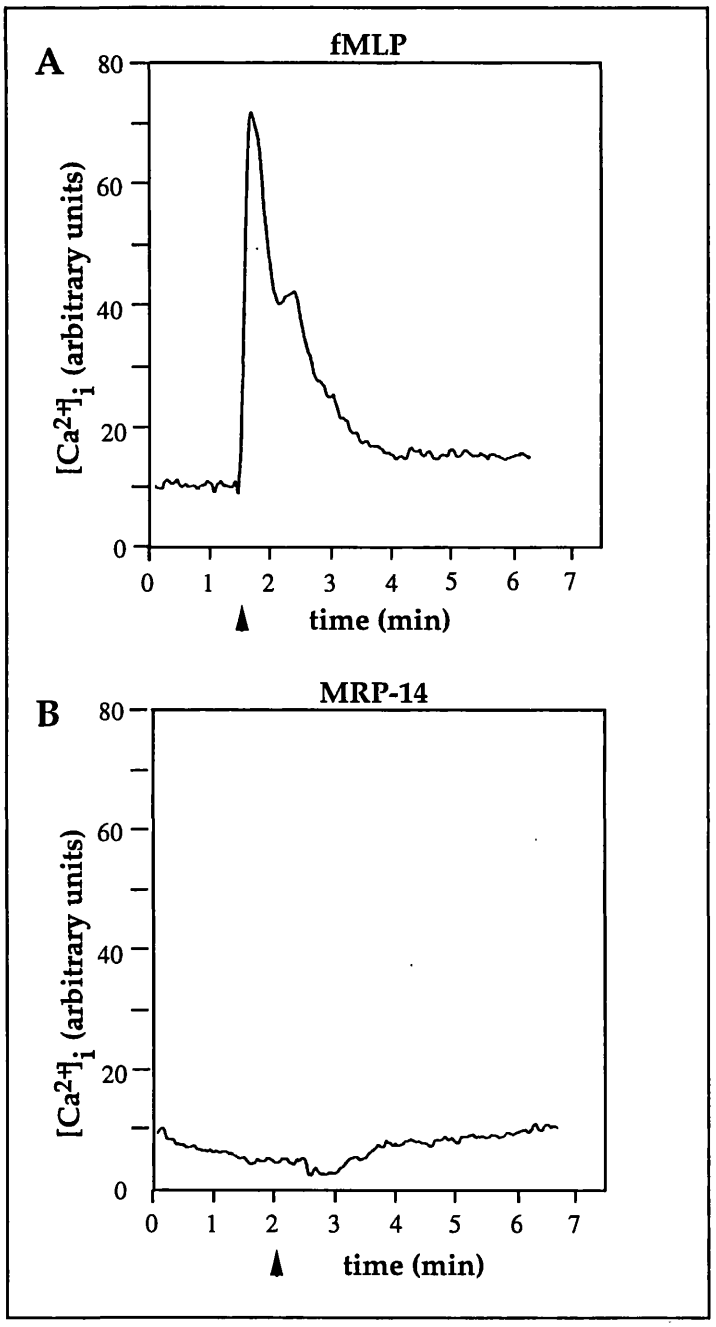

Figure 4.4: The effect of MRP-14 human neutrophil calcium flux. A. fMLP (0.1μM) but not **B.** MRP-14 (1μM) induces an intracellular calcium flux (n=6). The times of addition of the MRP-14 or fMLP to the neutrophils is indicated by the arrows on the Figure. Increases in $[Ca^{2+}]_i$ are detected by an increase in the fluorescence of FURA/2 and are represented in arbitrary units. Results are from one experiment representative of six.

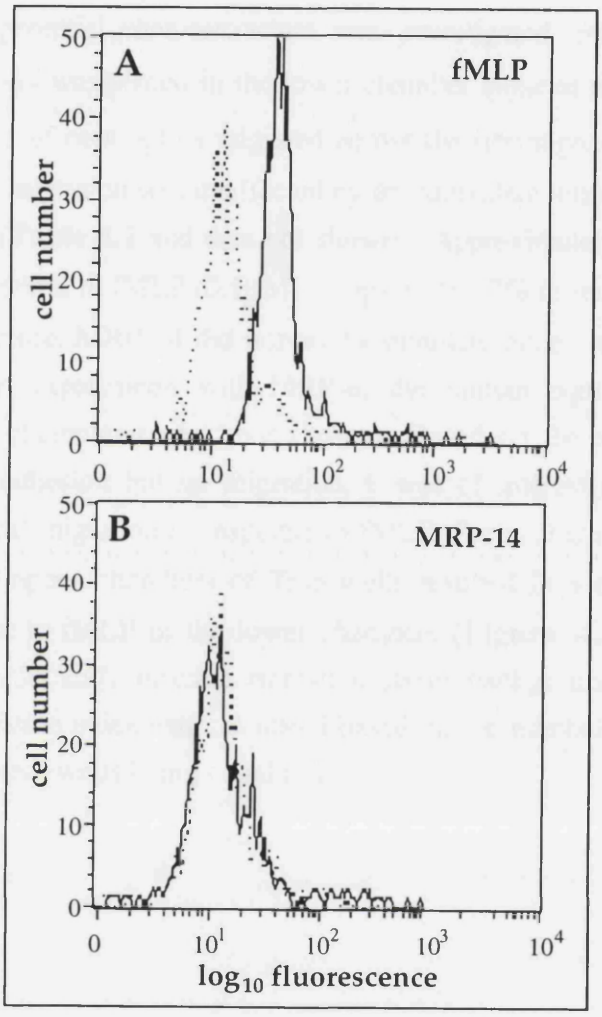

Figure 4.5: The effect of MRP-14 on neutrophil respiratory burst. A. fMLP (0.1μM) but not **B.** MRP-14 (1μM) induces neutrophil superoxide production as measured by an increase in dihydrorhodamine fluorescence (FL-1 channel) (n=3). Pretreatment levels of expression are indicated as (·····) and post treatment levels as (———).

4.2.3 THE EFFECT OF MRP-14 ON NEUTROPHIL MIGRATION

The possibility still existed that MRP-14, despite the absence of exocytosis, Ca^{2+} flux or superoxide production, could act as a neutrophil chemoattractant. For example, the murine MRP-8 homologue, CP-10, is reported to be chemotactic for neutrophils without inducing a Ca^{2+} flux (Cornish et al., 1996). The capacity of MRP-14 (up to 2μM) to act as a potential chemoattractant was investigated using the Transwell system. When MRP-14 was placed in the lower chamber alone at concentrations of ≥ 1μM, small numbers of neutrophils migrated across the fibrinogen-coated membrane and this low level of migration was unaffected by an equivalent amount of MRP-14 in the upper chamber (**Table 4.1** and data not shown). Approximately 70% of the total cells migrated in response to fMLP (0.1μM) compared to ~3% in response to MRP-14 (**Table 4.1**). Therefore, MRP-14 did not act to stimulate either random or directed migration. Similarly, experiments with MRP-8, the human equivalent of CP-10, proved negative for chemotaxis (data not shown). Based on the ability to stimulate high affinity Mac-1 adhesion but no migration, it was of interest to see if MRP-14 could effect neutrophil migration in response to fMLP. Preincubation of cells with 1 μM MRP-14 in the upper chambers of Transwells resulted in a decrease of ~50% migration in response to fMLP in the lower chambers (**Figure 4.6**). As expected, MRP-14 did not significantly increase migration above background (**Figure 4.6**). The migration stimulation index was calculated based on the number of migrated cells in control (unstimulated) wells being equal to 1.

TREATMENT	cells ($x\ 10^2$)±SD	% input cells above background
unstimulated	93.3 ± 43.2	-
MRP-14 in lower wells	233 ± 30.1	2.8
MRP-14 in upper wells	110 ± 21.0	0.3
MRP14 in upper/lower	233 ± 30.6	2.8
FMLP in lower wells	3550 ± 118	69

Table 4.1: The migration of human neutrophils through fibrinogen-coated Transwells in response to optimal concentrations of MRP-14 (1μM) and fMLP (0.1μM) (n=6). Result are expressed as the total number of cells migrated/well± standard deviations. Percentages of cells migrating above background were calculated from the total number of cells added/ well, i.e. 5×10^5.

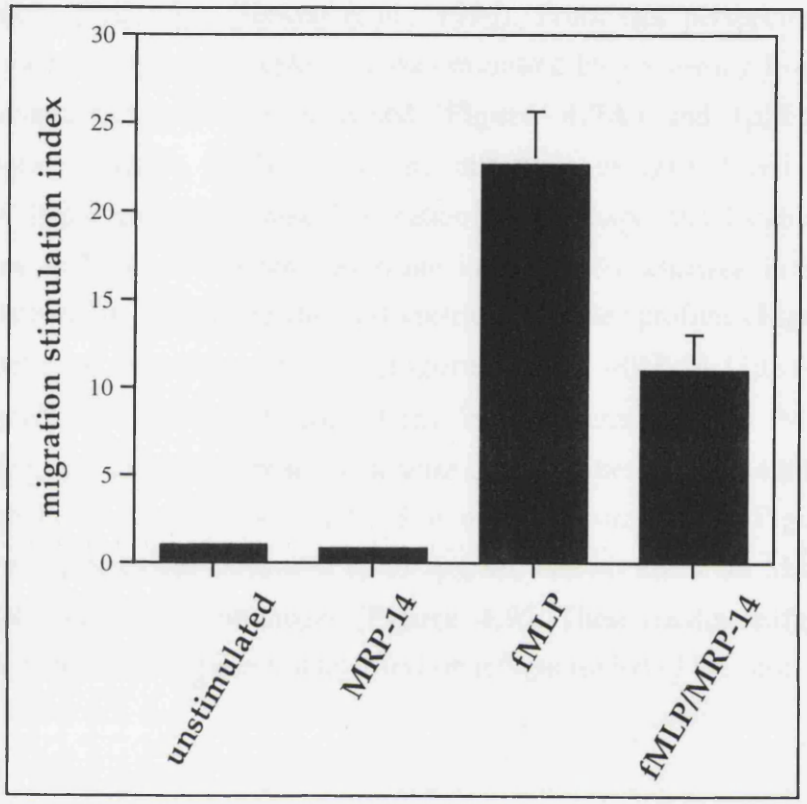

Figure 4.6: The effect of MRP-14 on neutrophil migration in response to fMLP. Neutrophils were preincubated with 1μM MRP-14 in the upper chambers of Transwells for 30 min at RT prior to addition of fMLP (0.1μM final concentration) to the lower wells and further incubation at 37°C for 45 min.. Controls for migration in response to buffer, MRP-14 or fMLP alone were also included. Migrated cells were quantitated by flow cytometry for quadruplicate samples and converted into a migration stimulation index, where unstimulated migration=1. The data shown are representative of three experiments.

4.2.4 THE EFFECT OF MRP-14 ON NEUTROPHIL MORPHOLOGY

From the data presented so far, MRP-14 function appeared to be limited specifically to increasing adhesive function of Mac-1 without general stimulation of other neutrophil functions or induction of chemotaxis. Integrin activation can be associated with remodelling of the cytoskeleton, with cell spreading considered to increase the efficiency of adhesion (Stewart et al., 1996). From this perspective, the effect of MRP-14 on neutrophil morphology was evaluated by visualising F-actin. A confocal microscopic comparison of untreated (**Figure 4.7A**) and 1μM MRP-14-treated neutrophils (**Figure 4.7B**) shows no difference in general cell shape, whereas 0.1μM fMLP causes substantial alteration of cell shape and localisation of F-actin (**Figure 4.7C**). An attempt was made to assess the changes in cell shape more quantitatively by comparing flow cytometric cell scatter profiles (**Figure 4.8A**) and the kinetics of induction of F-actin (**Figure 4.8B**). MRP-14 (1μM) failed to cause distinguishable cell shape change or formation of F-actin, whereas fMLP (0.1μM), as expected, was able to promote an increase in cell scatter (**Figure 4.8A**) and a 1.5 fold increase in neutrophil F-actin after 3 minutes of stimulation (**Figure 4.8B**). In addition, cytochalasin D, titrated up to 1μg/ml, had no effect on MRP-14-stimulated neutrophil adhesion to fibrinogen (**Figure 4.9**). These results confirmed that MRP-14 treatment of neutrophils had no effect on reorganisation of the actin cytoskeleton.

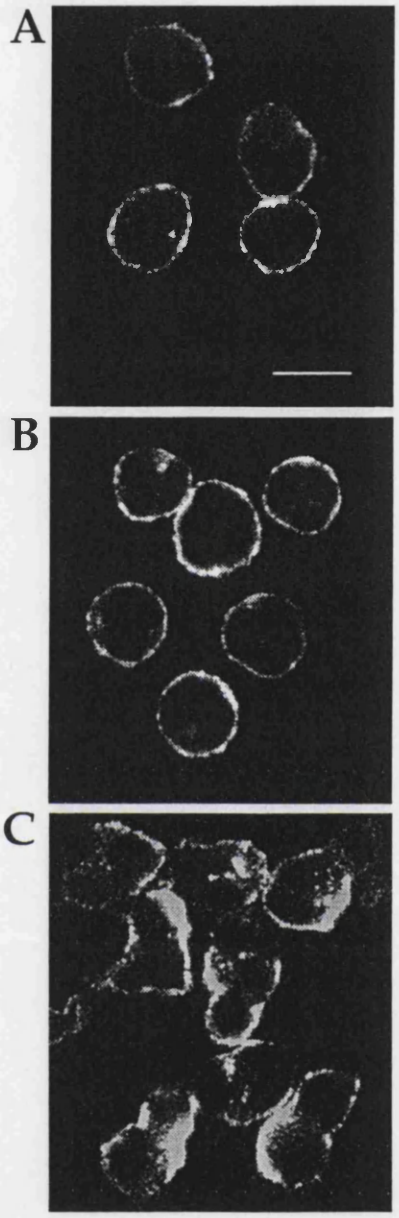

Figure 4.7: Confocal microscopic study of the effect of MRP-14 on neutrophil morphology. FITC-phalloidin staining of F-actin in **A.** unstimulated neutrophils or cells treated with **B.** MRP-14 (1μM) and, **C.** fMLP (0.1μM) (n=3). Bar=10μM. Note: In A. a field containing a greater than the average number of adherent unstimulated cells was chosen for comparison with stimulated cells.

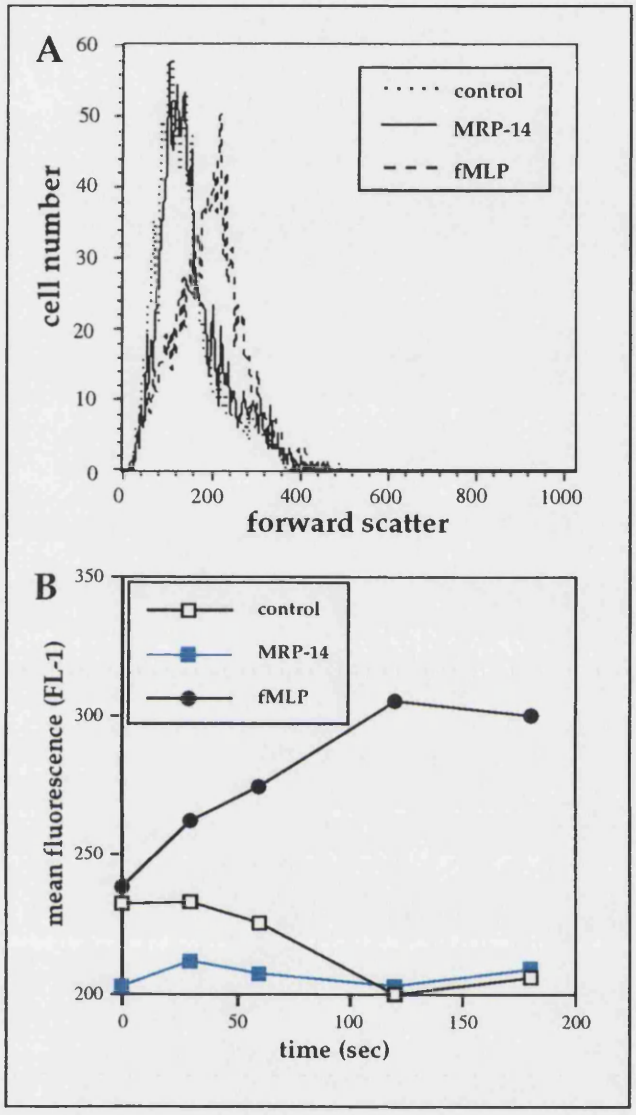

Figure 4.8: Flow cytometric measurements of the effect of MRP-14 and fMLP on A. cell scatter profiles and B. F-actin polymerisation. A. fMLP (0.1μM) (- - - - - -) but not MRP-14 (1μM) (————) induces an increase in forward scatter compared with unstimulated cells (·····) (n=6); **B.** fMLP (0.1μM) (—●—) but not MRP-14 (1μM) (—■—) increases FITC-phalloidin staining of permeabilised neutrophils over 3 min (n=3). Unstimulated cells are indicated as (—□—).

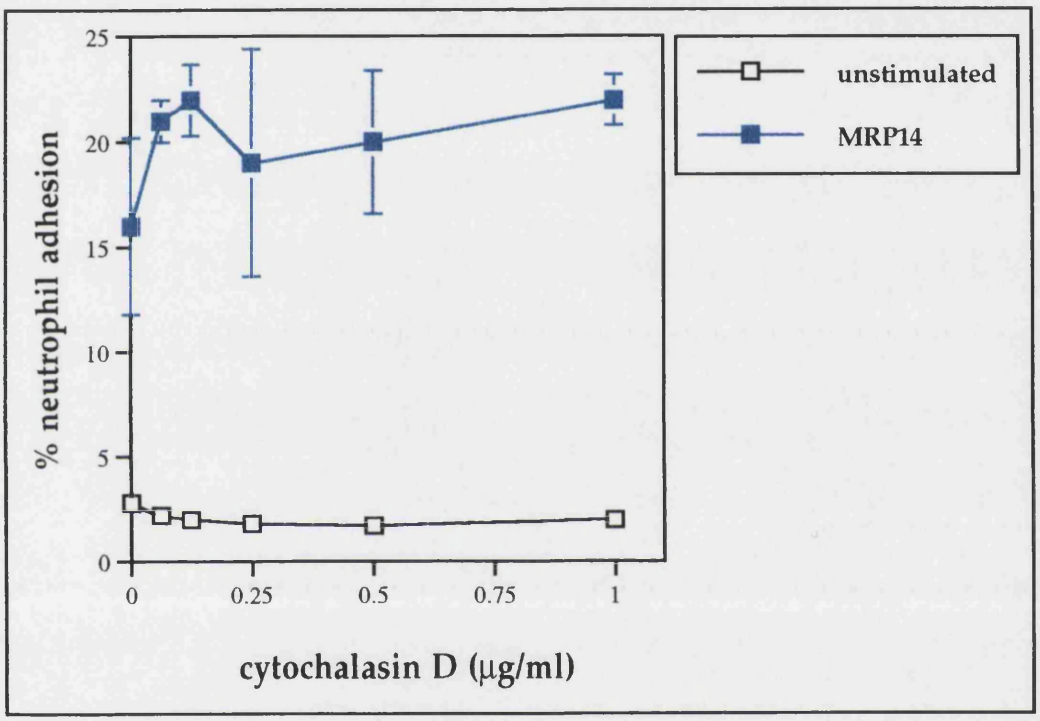

Figure 4.9: The effect of cytochalasin D on MRP-14-induced neutrophil adhesion to fibrinogen. Cytochalasin D, titrated from 0-1 µg/ml, was unable to inhibit the adhesion induced by 1µM MRP-14 (—■—). Unstimulated cell adhesion was similarly unaffected (—□—). Results are expressed as the mean % neutrophil adhesion ± standard deviation (n=3).

4.3 DISCUSSION

From the results in this chapter it can be concluded that MRP-14 affects the neutrophil adhesive state by directly inducing an increase in the affinity of Mac-1 on neutrophils. This is of interest because there has been a lack of identification of naturally occurring signalling molecules which regulate integrin function. The action of MRP-14 is very selective in that other markers of the activated neutrophil are not evident after treatment with this protein.

It is becoming apparent that integrin-mediated adhesion can occur by two distinct mechanisms. By one means, a combination of integrin redistribution into clusters on the cell membrane accompanied by accessory events such as cell spreading acts to increase the strength of adherence to ligand (reviewed in (Lub et al., 1995; Stewart and Hogg, 1996)). This form of adhesion is dependent on rearrangement of the actin cytoskeleton and is sensitive to cytochalasin D. There are fewer examples of a second mechanism which involves conformational alteration of integrin leading to enhanced affinity for ligand. This increase in ligand binding affinity can be brought about by artificial means such as treatment with agents such as divalent cations Mn^{2+} or Mg^{2+} and activating monoclonal antibodies (Stewart and Hogg, 1996). We provide here an example of affinity regulation of Mac-1 integrin by the naturally occurring S100 protein MRP-14, which triggers Mac-1 on neutrophils to bind soluble fibrinogen. High affinity status of Mac-1 is further confirmed by the ability of MRP-14 to cause expression of the mAb 24 activation reporter epitope, which also detects the Mg^{2+}-treated Mac-1 on neutrophils (Dransfield and Hogg, 1989). Therefore, MRP-14 represents one of few examples of a physiological protein acting as a very selective modulator of integrin affinity without activating neutrophil effector functions. Another example of affinity regulation of Mac-1 follows from the treatment of monocytes with ADP which induces Mac-1-mediated binding of soluble ligand fibrinogen (Altieri et al., 1988) and Factor X (Altieri and Edgington, 1988). Also L-selectin cross-linking or ligation with ligand causes the expression of the mAb 24 epitope on neutrophils and lymphocytes (Hwang et al., 1996; Simon et al., 1995).

The affinity change to Mac-1 in response to MRP-14 occurs in the absence of accessory adhesion events such as neutrophil shape change, actin reorganisation or cell spreading, underlining the restricted scope of the signalling activity of MRP-14. As increases in intracellular Ca^{2+} facilitate adhesion through cell spreading for integrins such as LFA-1 (Stewart et al., 1996), the fact that Mac-1 adhesion is evident in the absence of an increase in $[Ca^{2+}]_i$ is a further indication that these accessory events are not part of the mechanism of MRP-14 action. Finally, the lack of sensitivity to

cytochalasin D of MRP-14-stimulated neutrophil adhesion is additional proof that it is integrin itself which is altered and that the cytoskeleton has no role in MRP-14 function.

The failure of MRP-14 to cause loss of L-selectin, Mac-1 upregulation and Ca^{2+} flux on neutrophils resembles the functional profile of the murine chemotactic S100 protein CP-10 (Cornish et al., 1996). In contrast, the failure of MRP-14 to induce shape change, actin reorganisation or neutrophil migration indicates that MRP-14 does not act as a chemoattractant. Furthermore, human MRP-8 is not chemotactic, unlike the murine homologue ((Lackmann et al., 1993) and data not shown). This lack of chemotactic activity of MRP-8 and MRP-14 contrasts with CP-10 (Lackmann et al., 1992) and the two other chemotactic S100 proteins, S100L (Komada et al., 1996) and psoriasin (Jinquan et al., 1996). None of these proteins have yet been tested for their ability to directly activate integrins but it can be concluded that functional differences exist between this subset of S100 family members, which operate within the context of an immune response.

The fact that MRP-14 does not recruit new Mac-1 to the membrane is not detrimental to function because it is the constitutively expressed Mac-1 which is responsive to adhesion activating stimuli (Buyon et al., 1988). When neutrophils are stimulated with classical chemoattractants, such as fMLP, the total expression of Mac-1 on the membrane can increase by up to ten fold (Todd III et al., 1984) but this newly arrived Mac-1 is inactive until a second round of stimulation (Hughes et al., 1992). If MRP-14 does supply the signal for activation of β_2 integrin adhesion then it may be able to influence other events of the adhesion cascade. For example, the failure to activate L-selectin shedding could have a positive effect on neutrophil accumulation at sites of leukocyte trafficking. L-selectin shedding has been considered necessary for effective neutrophil rolling and tethering on the endothelium, but prevention of L-selectin cleavage causes neutrophils to roll more slowly, bringing them into close contact with the adhesive surface (Walcheck et al., 1996). Furthermore, under flow conditions, adhering neutrophils will recruit further neutrophils through L-selectin-mediated adhesion between neutrophils. This route of enhanced binding is eliminated if L-selectin is shed through neutrophil activation (Kuijper et al., 1997). As well as directly influencing the above adhesion events, other classical chemoattractants, such as fMLP and the chemokines serve as general neutrophil activators inducing functions such as Ca^{2+} flux, the respiratory burst and degranulation (see (Baggiolini et al., 1994)). Adhering neutrophils with an activated respiratory burst release harmful products such as hydrogen peroxide and oxygen radicals (Nathan et al., 1989) which damage endothelium and surrounding tissue (Vedder et al., 1990). The restricted action of

MRP-14 may be beneficial in minimising the potential damage which neutrophils could inflict on vascular endothelium. The results presented in this chapter, considered together, suggest that MRP-14 represents a new candidate for *in vivo* activation of the Mac-1 β_2 integrin. MRP-14 is unique from the other candidates described as it is able to directly activate high affinity Mac-1 without stimulating other neutrophil effector functions.

THE MECHANISM OF MRP-14 ACTION ON NEUTROPHILS

5.1 INTRODUCTION

As discussed in the previous chapter, neutrophil adhesion can occur through two distinct mechanisms of integrin activation; by direct binding to the integrin or through intracellular signals generated by engagement of other cell surface receptors. To date only certain divalent cations, such as Mg^{2+} (Dransfield et al., 1992) or Mn^{2+} (Altieri, 1991), activating antibodies (Andrew et al., 1993; Petruzzelli et al., 1995; Robinson et al., 1992) and an endogenous neutrophil lipid factor (Hermanowski-Vosatka et al., 1992; Klugewitz et al., 1997) have been described to activate neutrophil adhesion by direct binding to Mac-1. Adhesion to fibrinogen has also been demonstrated upon physical association of Mac-1 with other cell surface receptors, such as the GPI-linked urokinase receptor (uPAR, CD87) on monocytic cells (Simon et al., 1996).

In contrast, adhesion induced via "inside out" signalling in neutrophils has been described for chemoattractants, cytokines, growth factors, L-selectin cross-linking, adenosine nucleotides and phorbol esters (see Introduction and Chapter 4). The engagement of neutrophil receptors for these factors result in multiple neutrophil effector functions, in addition to adhesion, such as Mac-1 upregulation, respiratory burst, degranulation, Ca^{2+} mobilisation and migration (described in Chapter 4 and reviewed in (Baggiolini et al., 1994)). The intracellular signaling pathways involved in stimulating these neutrophil effector functions have been aided by the studies of chemoattractants, such as fMLP, C5a and IL-8, which interact with G protein-coupled receptors on the neutrophil membrane (see below). In these studies, apart from the requirement for G protein activation, adhesion in response to chemoattractants has been shown to be dependent on activation of various intracellular enzymes, such as tyrosine kinases (Naccache et al., 1994), phospholipase A_2 (Jacobson and Schrier, 1993), phosphatidylinositol (PI) 3-kinase (Knall et al., 1996; Metzner et al., 1997), and MAP kinase (Knall et al., 1996; Nick et al., 1997). In addition, the small GTP-binding protein Rho has also been shown to be central to chemoattractant-induced neutrophil adhesion (Laudanna et al., 1996). Due to the selective response of neutrophils to MRP-14, it was of interest to determine whether MRP-14 was able to bind directly to Mac-1 and induce ligand binding, or whether Mac-1 activation on

neutrophils was due to signals generated through interaction of MRP-14 with a distinct receptor on the neutrophil membrane. Furthermore, if MRP-14 is able to signal through a distinct receptor, then it is also of interest to investigate the nature of the signals generated in reference to those described for chemoattractant activation of neutrophil Mac-1 adhesion.

5.2 RESULTS

5.2.1 MRP-14 BINDS TO A DISTINCT NEUTROPHIL RECEPTOR

In order to determine the means by which MRP-14 altered the function of Mac-1, the interaction of MRP-14 with the neutrophil membrane was first investigated. FITC-MRP-14, titrated from 0-4μM, was found to bind to a specific site on the neutrophil membrane in a dose-dependent and cation-dependent manner (**Figure 5.1**). The binding, although not saturated at 4μM, was MRP-14 specific, as all FITC-MRP-14 binding could be blocked by the addition of unlabelled MRP-14, but not by the addition of control S100A protein (**Figure 5.2**). In order to investigate the possibility that the MRP-14 receptor was Mac-1 itself, the MRP-14 binding profiles of Mac-1 K562 transfectant (KC/16 cells) by comparison with the parent K562 cells (Annenkov et al., 1996; Ortlepp et al., 1995) were tested. There was no difference in the ability of KC/16 cells to bind FITC-MRP-14, compared with non-Mac-1-expressing K562 cells (**Figure 5.3A**), even though the KC/16 cells abundantly expressed Mac-1 (**Figure 5.3B**). The FITC-MRP-14 binding to both K562 and KC/16 cells was also prevented by unlabelled MRP-14, but not by S100A protein (data not shown), further supporting the specificity of MRP-14 binding. In addition, no evidence was obtained for co-precipitation of MRP-14 and Mac-1 from neutrophil membranes, using an anti-MRP-14 specific mAb (data not shown). Furthermore, no tested Mac-1 mAb, including the blocking mAbs 2LPM19c, 2H3, and OKM1, and the non-blocking mAb 44, inhibited the binding of MRP-14 to neutrophils (data not shown). The conclusion from these experiments is that MRP-14 binds to a receptor distinct from Mac-1 on the surface of neutrophils.

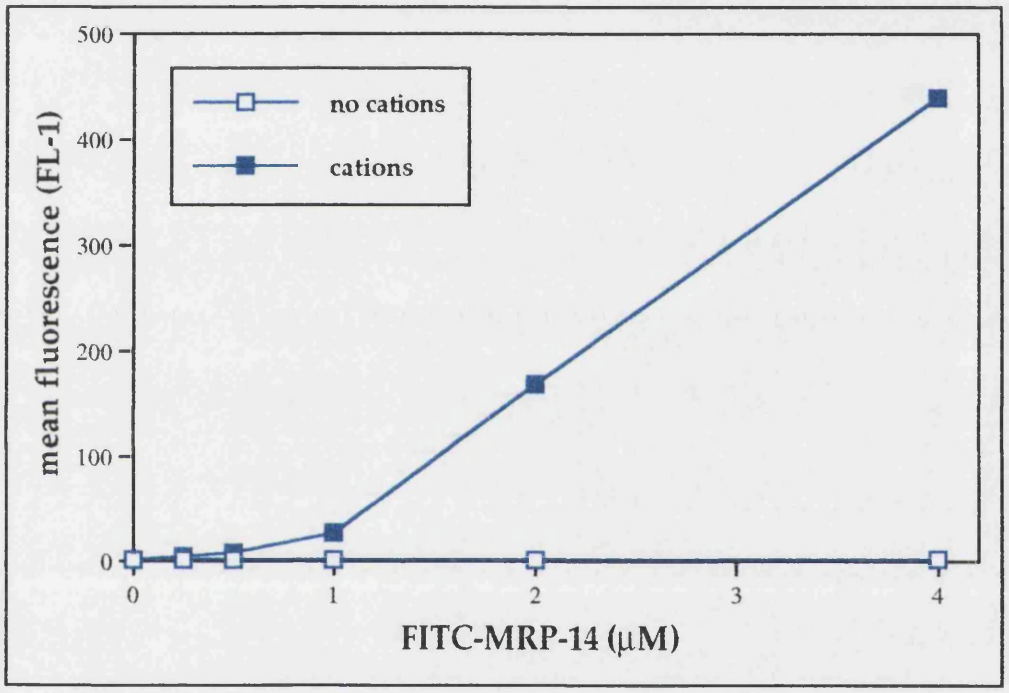

Figure 5.1: MRP-14 binds directly to the neutrophil membrane. FITC-MRP-14, titrated from 0-4μM, binds to the neutrophil membrane in a dose-dependent manner in the presence (—■—) but not in the absence (—□—) of 1mM Ca^{2+}, 1mM Mg^{2+},and 10μM Zn^{2+}. Data are from one experiment representative of six.

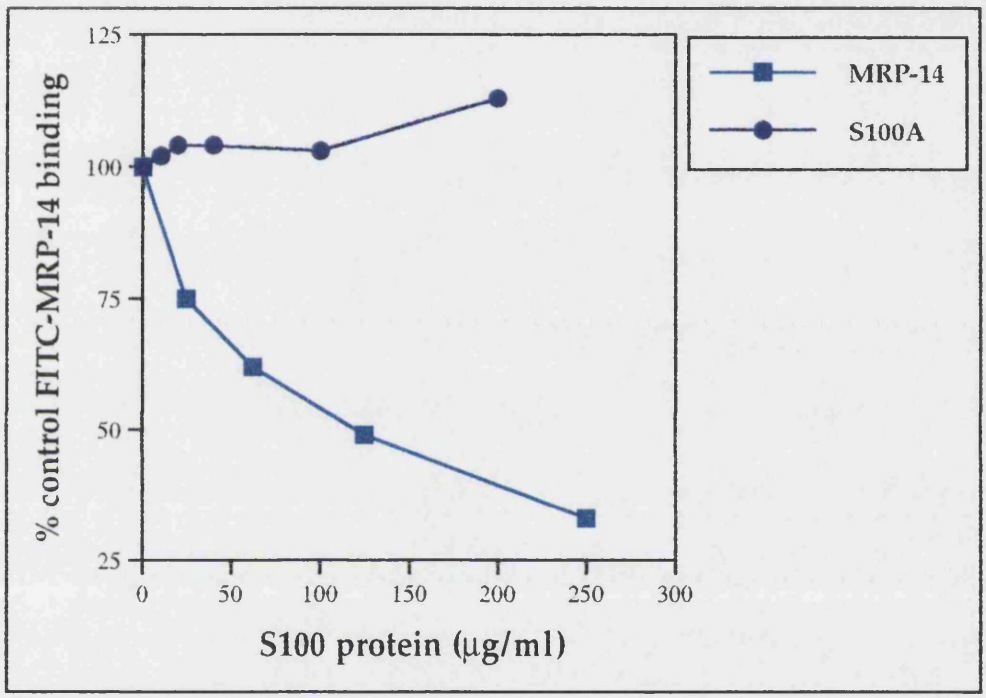

Figure 5.2: The effect of S100 proteins on MRP-14 binding to the neutrophil membrane. FITC-MRP-14 (25µg/ml) binding to neutrophils is specifically inhibited by unlabelled MRP-14 (—■—), but not by control S100A protein (—●—). Data presented are single samples representative of five experiments.

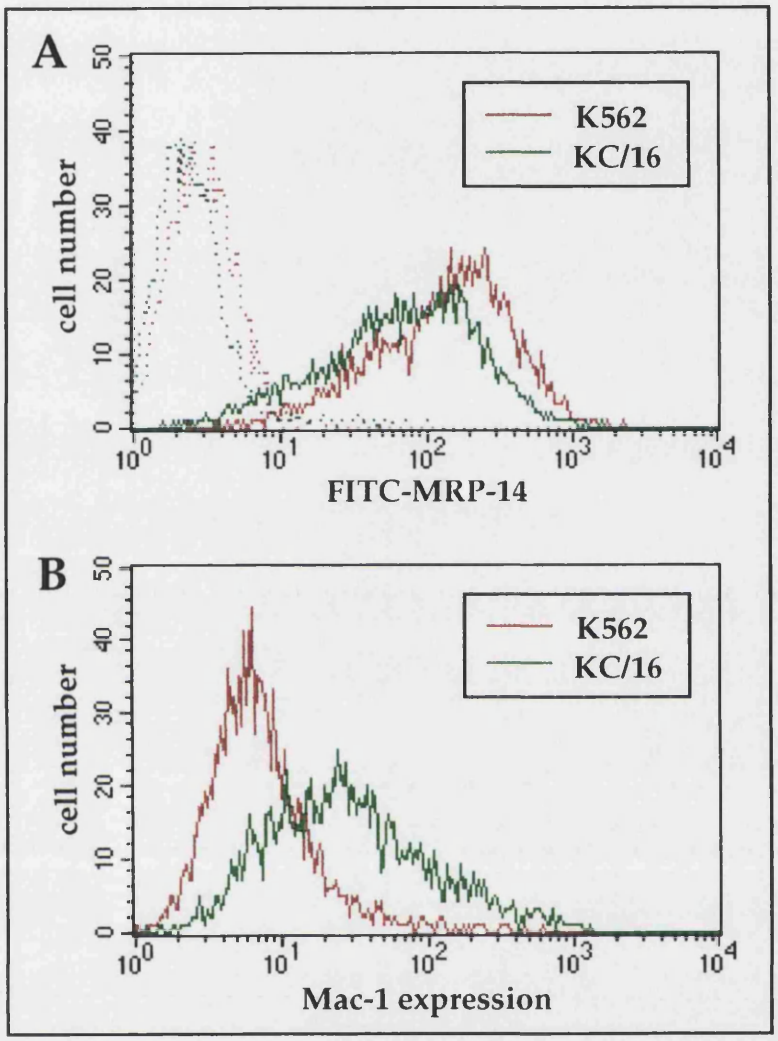

Figure 5.3: The binding of MRP-14 to K562 cells. A. FITC-MRP-14 (2μM) binds to Mac-1 transfected (KC/16) (———) and untransfected (———) K562 cells. Unstained KC/16 and K562 are represented as (- - - - - -) and (- - - - - -) **B.** As a control the Mac-1 expression levels on KC/16 cells are shown (———), compared to untransfected, Mac-1 negative, K562 cells (———). The profiles shown are from one representative experiment of four.

5.2.2 MRP-14 BINDS TO A PERTUSSIS TOXIN-SENSITIVE RECEPTOR

In addition to chemoattractants, two other S100 proteins have been demonstrated to bind to pertussis toxin-sensitive G protein-coupled receptors (Cornish et al., 1996; Komada et al., 1996). To aid understanding of the function of MRP-14, the possibility that MRP-14 also operated through a G protein-coupled receptor was investigated. Neutrophils preincubated with pertussis toxin showed a concentration-dependent decrease in the ability of MRP-14 to stimulate adhesion (**Figure 5.4**). FMLP-stimulated adhesion was also inhibited, as expected; however, the inhibition was only ~50% with 1μg/ml pertussis toxin (**Figure 5.4**). MRP-14-stimulated adhesion, in contrast, was fully inhibited to background levels at the same concentration of pertussis toxin. In addition to the adhesion induced by MRP-14 being inhibited by pertussis toxin, MRP-14-induced mAb 24 epitope expression and, therefore, Mac-1 activation, was also inhibited (**Figure 5.5**). As for the results of the fibrinogen binding assay, pertussis toxin at 1μg/ml inhibited the mAb 24 epitope expression induced by MRP-14 and fMLP (**Figure 5.5A and B**). As for Figure 5.4, MRP-14-induced mAb 24 expression (and therefore integrin activation) was inhibited to a greater extent than that induced by fMLP. As pertussis toxin selectively inhibits the α_i G protein subunit, these results imply that fMLP receptors may be coupled to additional G proteins which can also signal integrin activation. In contrast, the mAb 24 epitope expression stimulated with the phorbol ester, PMA, whose receptor is not G protein-coupled, was not pertussis toxin-sensitive (**Figure 5.5C**). Together these findings suggest that MRP-14 interacts with neutrophils via a G protein-coupled receptor and that the subsequent signalling leads to Mac-1 activation.

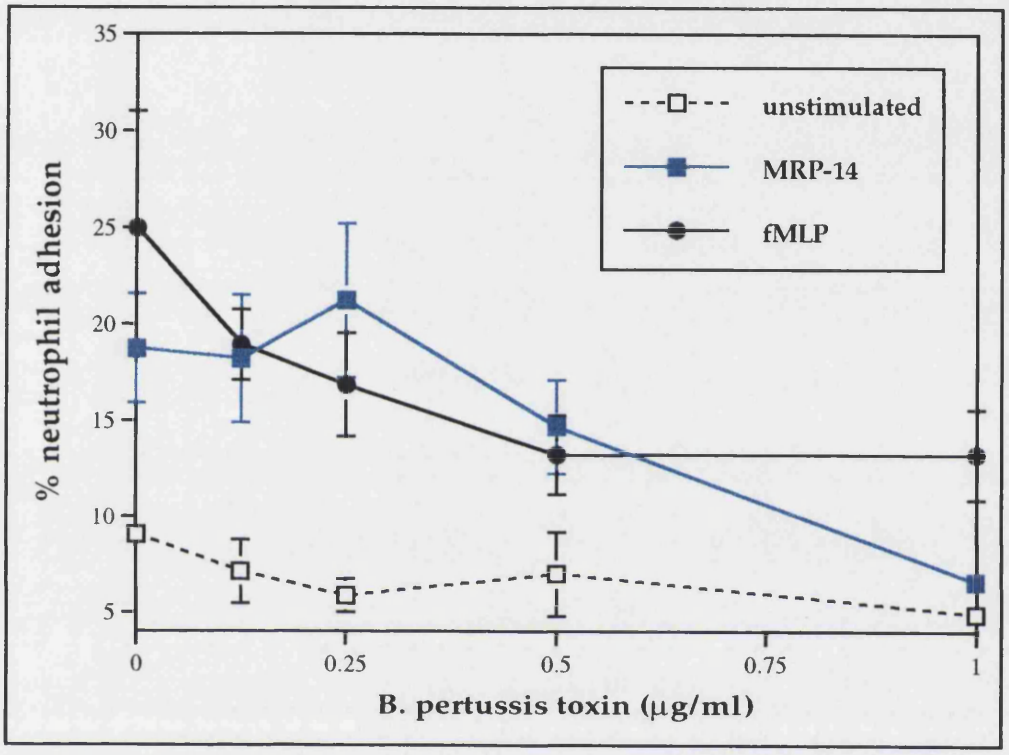

Figure 5.4: Pertussis toxin sensitivity of MRP-14 and fMLP adhesion. The adhesion induced by both MRP-14 and fMLP is sensitive to Bordetella pertussis toxin, titrated from 0-1.0 µg/ml inhibited the adhesion induced by 1µM MRP-14 (—■—) and 0.1µM fMLP (—●—), without affecting unstimulated neutrophil binding (- - -□- - -). These concentrations of pertussis toxin had no effect on neutrophil viability. Results are expressed as the mean % neutrophil adhesion ± standard deviation. The data illustrated show one experiment of five.

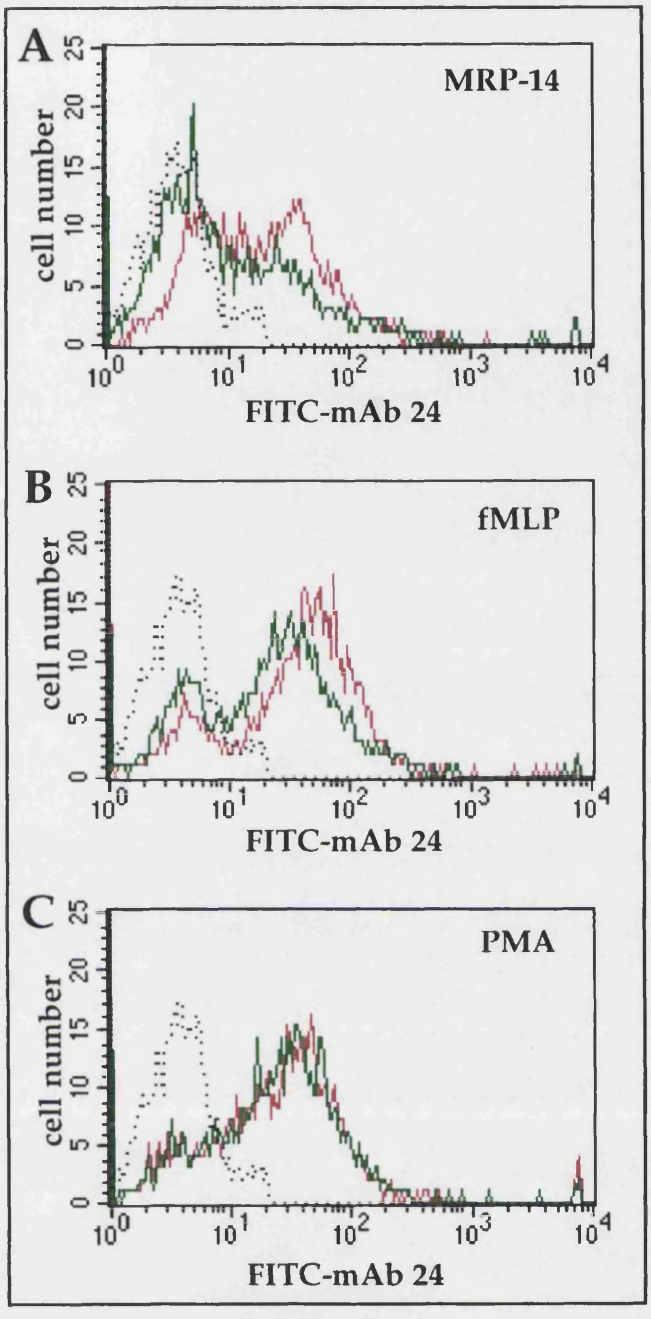

Figure 5.5: The effect of pertussis toxin on β_2 integrin activation by stimulators of neutrophil adhesion. Pertussis toxin at 1μg/ml inhibited the expression of the β_2 integrin activation epitope, recognised by mAb 24, on cells stimulated with **A.** 2μM MRP-14 and **B.** 0.1μM fMLP. **C.** In contrast, the expression of the mAb 24 epitope on PMA-stimulated neutrophils was unaffected by preincubation with pertussis toxin. Unstimulated mAb 24 expression is as (·····). Glycerol control preincubated cells are indicated as (———) and pertussis toxin-treated cells as (———). The data shown are from one representative experiment of four.

5.2.3 A ROLE FOR PHOSPHATIDYLINOSITOL 3-KINASE IN MRP-14 RECEPTOR SIGNALLING

Since MRP-14 also appeared to operate through a G protein-coupled receptor, it was of interest to determine if the signalling pathways activated by other G protein-coupled receptors were activated by MRP-14. To investigate these possibilities, the effect of signalling inhibitors on the expression of the β_2 integrin activation epitope (recognised by mAb 24) was tested. The studies described in this chapter have included only those inhibitors previously described as inhibitory to G protein-stimulated adhesion. The inhibitors used are described in the Materials and Methods chapter.

Recently, the ability of chemoattractants to stimulate neutrophil adhesion was shown to be sensitive to the phosphatidylinositol (PI)-3-kinase inhibitors wortmannin and LY294002 (Knall et al., 1996; Metzner et al., 1997). In keeping with this, neutrophils preincubated with 50nM wortmannin were less responsive to MRP-14, as determined by reduced mAb 24 expression (**Figure 5.6A**). This inhibition was detected at concentrations of wortmannin as low as 5nM and was maximal at 50nM (**Figure 5.6A** and data not shown). As predicted from the previous reports (Metzner et al., 1997), fMLP activation of mAb 24 expression was also inhibited by 50nM wortmannin (**Figure 5.6B**). In contrast, the response to the PKC activator, PMA, was unaffected (**Figure 5.6C**). These concentrations are in keeping with inhibition of PI 3-kinase (Arcaro and Wymann, 1993), as opposed to myosin light chain kinase, which is inhibited at 10-100 fold higher concentrations of wortmannin (Nakanishi et al., 1994). To confirm that PI 3-kinase, and not another related kinase, was being inhibited in Figure 5.6, neutrophils were preincubated with the other PI 3-kinase inhibitor, LY294002 (Vlahos et al., 1995). MRP-14 activation of mAb 24 expression was similarly inhibited with 10μM LY294002 (**Figure 5.7A**). In agreement with results for wortmannin, fMLP was also inhibited by LY294002 at 10μM (**Figure 5.7B**). PMA stimulation of mAb 24 expression was unaffected by the preincubation with LY294002, confirming the lack of PI 3-kinase involvement in β_2 integrin activation by phorbol ester (**Figure 5.7C**). In these experiments the level of mAb 24 expression on unstimulated cells was unaffected by the PI 3-kinase inhibitors (data not shown). In addition, neither wortmannin or LY 294002 had any affect on cell viability, as determined by Trypan Blue exclusion (data not shown).

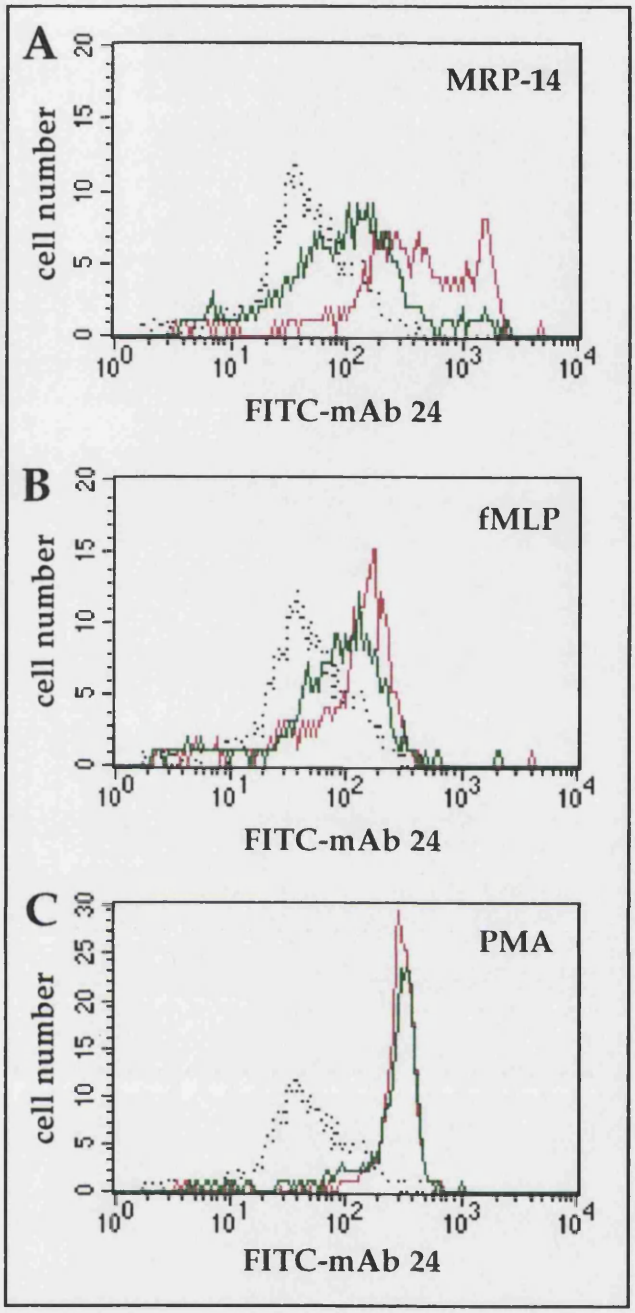

Figure 5.6: MAb 24 expression on MRP-14-treated neutrophils is sensitive to the PI 3-kinase inhibitor wortmannin. A. MAb 24 expression on neutrophils treated with 2μM MRP-14 is inhibited by preincubation of the cells with 50nM wortmannin. **B.** Similarly, fMLP-induced mAb 24 expression is also inhibited. **C.** In contrast, the expression of the mAb 24 epitope on PMA-stimulated neutrophils was unaffected by preincubation with wortmannin. Unstimulated mAb 24 expression is as (·····). DMSO control preincubated cells are indicated as (———) and wortmannin-treated cells as (———). The data shown are from one representative experiment of four.

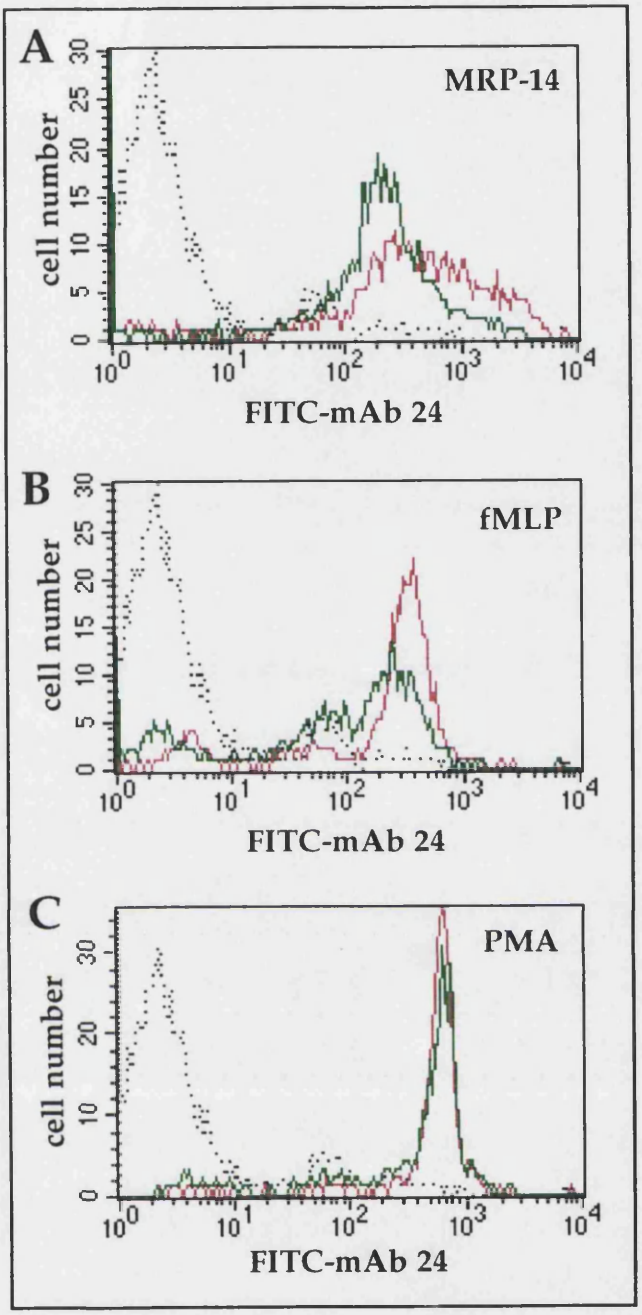

Figure 5.7: MAb 24 expression on MRP-14-treated neutrophils is also sensitive to the PI 3-kinase inhibitor LY294002. A. MAb 24 expression on neutrophils treated with 2μM MRP-14 is inhibited by preincubation of the cells with 10μM LY294002. In contrast, the expression of the mAb 24 epitope on **B.** fMLP- and **C.** PMA-stimulated neutrophils was unaffected by preincubation with LY294002. Unstimulated mAb 24 expression is as (·····). DMSO control preincubated cells are indicated as (———) and LY294002-treated cells as (———). The data shown are from one representative experiment of four.

5.2.4 THE ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE IN MRP-14 RECEPTOR SIGNALLING

Evidence suggests that chemotactic peptides, such as fMLP and IL-8 can activate p42/44 MAP kinase through the sequential activation of PI 3-kinase, either MEK kinase (MEKK) (Avdi et al., 1996; Grinstein et al., 1994; Knall et al., 1996) or Raf (Worthen et al., 1994), and MAP kinase kinase (MEK). In addition, fMLP and PAF have been shown to induce Mac-1 adhesion via an unknown pathway which is partially dependent on p38 MAPK activation (Nick et al., 1997). The ability of MRP-14 to stimulate Mac-1 activation through PI 3-kinase, as seen in Figures 5.6A and 5.7A, prompted experiments to look at the downstream activation of MEK. These experiments were performed using the MEK inhibitor, PD098059 (Alessi et al., 1995). PD098059 was titrated from 0-100μM for its ability to inhibit the expression of the mAb 24 epitope. MRP-14-stimulated mAb 24 expression was inhibited by PD098059 with maximum inhibition occurring at 25μM of the MEK inhibitor (**Figure 5.8A**), suggesting that MEK (and possibly MAP kinase) activation is involved in Mac-1 activation. MAb 24 expression in response to fMLP was similarly inhibited, confirming the involvement of MAPK in fMLP receptor signalling the activation of β_2 integrins (**Figure 5.8B**). In addition, the activation of β_2 integrins by PMA was partially inhibited at 25μM PD098059 (**Figure 5.8C**). This result implies that PKC can also activate MEK to generate activated β_2 integrins. Again, unstimulated cell mAb 24 expression and neutrophil viability were unaffected by the MEK inhibitor (data not shown).

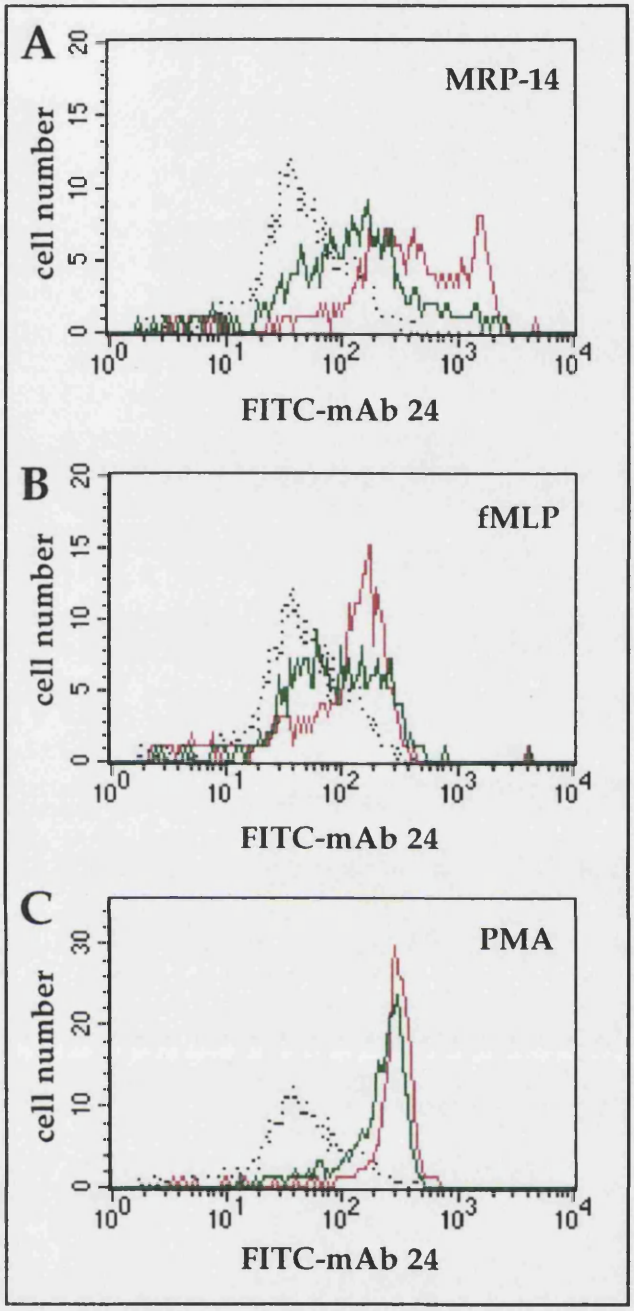

Figure 5.8: MAb 24 expression on MRP-14-treated neutrophils is sensitive to the MEK inhibitor PD098059. A. MAb 24 expression on neutrophils treated with 2μM MRP-14 is inhibited by preincubation of the cells with 25μM PD098059. **B.** Similarly, fMLP- and **C.** PMA-induced mAb 24 expression is also inhibited. Unstimulated mAb 24 expression is as (·····). DMSO control preincubated cells are indicated as (———) and PD098059-treated cells as (———). The data shown are from one representative experiment of four.

5.2.5 THE ROLE OF PROTEIN KINASE C IN MRP-14 FUNCTION

Activation of protein kinase C (PKC) accompanies, or is a direct result of, a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Activation of PKC has been shown to be responsible for activation of superoxide production in neutrophils in response to chemoattractants (Ahmed et al., 1995). Since neither increases in $[Ca^{2+}]_i$ nor superoxide production are evident in MRP-14 treated-neutrophils, it seemed unlikely that MRP-14 would stimulate PKC. Experiments were nevertheless performed to test the effect of the PKC inhibitor, *bis*-indolylmaleimide, on mAb 24 expression (Kiss et al., 1995). *Bis*-indolylmaleimide (2μM) was unable to inhibit the mAb 24 expression induced by MRP-14 (**Figure 5.9A**), but was able to partially inhibit the response to fMLP (**Figure 5.9B**) and fully inhibit the response to PMA (**Figure 5.9C**). These results confirm the lack of involvement of PKC in MRP-14 Mac-1 activation.

5.2.6 THE EFFECT OF SIGNALLING INHIBITORS ON MRP-14-STIMULATED NEUTROPHIL ADHESION TO FIBRINOGEN

As confirmation of the results seen above, the ability of signalling inhibitors to inhibit the adhesion to fibrinogen in response to MRP-14 was tested. As expected, wortmannin inhibited adhesion of neutrophils in response to MRP-14 (**Figure 5.10**) with ~50% of adhesion inhibition at 50nM wortmannin. Similarly, the MEK inhibitor, PD09859 at 25μM inhibited about ~50% of adhesion. The PKC inhibitor *bis*-indolylmaleimide had no effect on MRP-14 stimulated adhesion, which is in agreement with the results of Figure 5.9A. In addition to the inhibitors described in Figures 5.6-5.9, the protein tyrosine kinase inhibitor herbimycin A was included in the adhesion assay (**Figure 5.10**). In the presence of 10μg/ml herbimycin A, all adhesion was abolished, suggesting that MRP-14 activates tyrosine kinases in the neutrophil. However, in experiments of mAb 24 inhibition the role of protein tyrosine kinases in MRP-14 function is still unclear as in herbimycin A inhibited the unstimulated, as well as the stimulated, mAb 24 expression (**Figure 5.11**), without affecting cell viability. Thus it can be concluded, from the results described above, that MRP-14 appears to activate both PI 3-kinase and MEK, in the absence of any PKC activation and most likely involves protein tyrosine kinase activation.

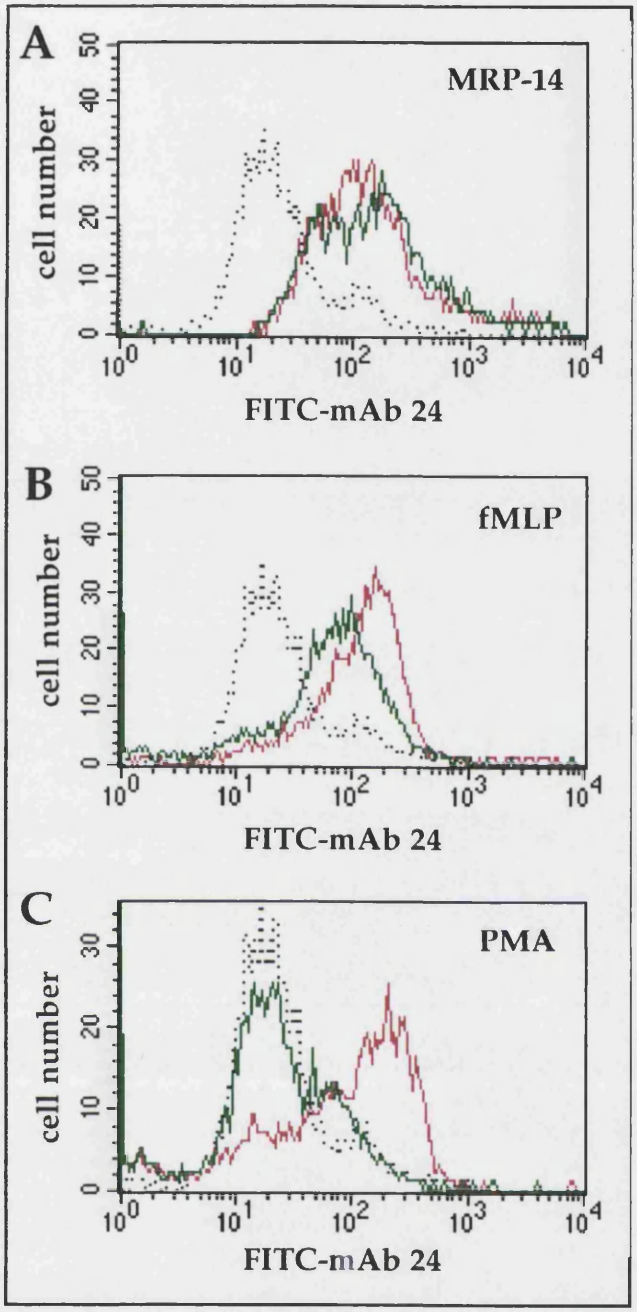

Figure 5.9: MAb 24 expression on MRP-14-treated neutrophils is insensitive to inhibition of protein kinase C. A. MAb 24 expression on neutrophils treated with 2μM MRP-14 is unaffected by preincubation of the cells with 2μM *bis*-indolylmaleimide. **B.** In contrast, fMLP-stimulated mAb 24 expression is partially inhibited and **C.** PMA-induced mAb 24 expression is fully inhibited. Unstimulated mAb 24 expression is as (·····). DMSO control preincubated cells are indicated as (———) and *bis*-indolylmaleimide-treated cells as (———). The data shown are from one representative experiment of four.

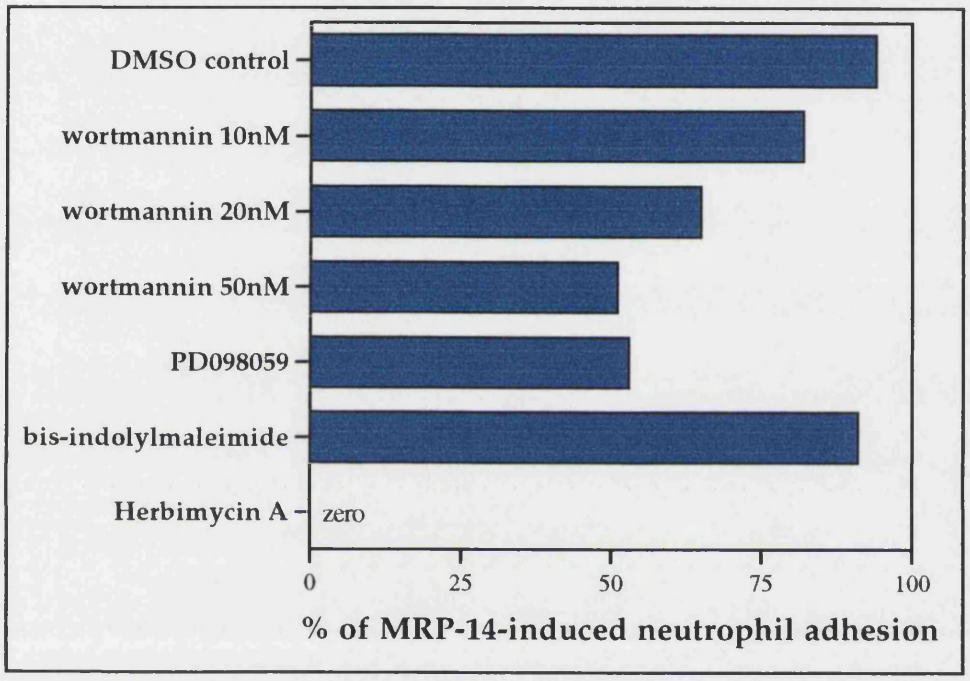

Figure 5.10: The effect of signalling inhibitors on neutrophil adhesion to fibrinogen stimulated by MRP-14. Cells were preincubated with inhibitors or DMSO controls for 30 min prior to activation with MRP-14 for a further 30 min. MRP-14 binding was sensitive to inhibitors of PI 3-kinase (wortmannin) and MEK (PD098059), but not to the inhibitor of PKC, *bis*-indolylmaleimide. Results are expressed as the mean % of MRP-14-induced neutrophil adhesion with no inhibitor/DMSO (=100%). The data are from duplicate wells and from one experiment of two.

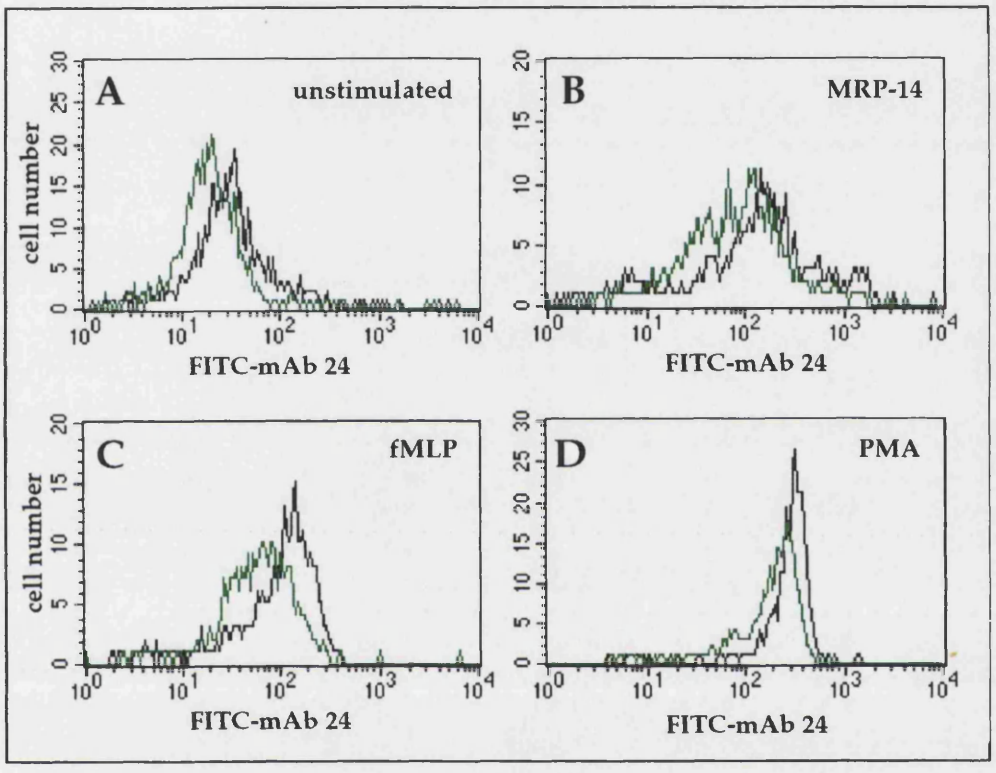

Figure 5.11: The effect of tyrosine kinase inhibition on mAb 24 expression on neutrophils. MAb 24 expression on **A.** unstimulated neutrophils or those treated with **B.** 2μM MRP-14 **C.** 0.1μM fMLP **D.** 50nM PMA is inhibited by 60 min preincubation of the cells with 10μg/ml herbimycin A. DMSO control preincubated cells are indicated as (———) and herbimycin A-treated cells as (———). The data shown are from one representative experiment of four.

5.3 DISCUSSION

The limited activity of MRP-14 for modulating integrin affinity, in the absence of stimulating other neutrophil effector functions, raised the question as to whether MRP-14 was binding directly to Mac-1. MRP-14 was found to bind to a specific receptor on neutrophils, however, this receptor was not saturable at 4μM MRP-14. The reasons for this lack of saturation are unknown but may be the result of aggregation of the protein upon FITC-conjugation, reducing the molecules available for binding. This would seem unlikely as FITC-MRP-14 when compared with unconjugated MRP-14 showed an identical titration curve for adhesion. An alternative explanation is that, binding may be approaching saturation at 2μM MRP-14 (**Figure 5.1**) and the binding at 4μM MRP-14 represents binding to a second lower affinity or non-specific site. Despite the specificity of the interaction shown by inhibition with unlabelled MRP-14, the possibility that MRP-14 stimulated Mac-1 function by direct binding to Mac-1 still remained. The evidence against such a mechanism of direct interaction of MRP-14 with Mac-1 was provided by MRP-14 binding to Mac-1 transfected and untransfected K562 cells. Binding was equivalent and specific for both the Mac-1 expressing and non-expressing cells. The fact that all of the proteins previously described to bind Mac-1 do so at/near the adhesive ligand binding site and can be competed for by the adhesive ligands such as fibrinogen further supports the lack of MRP-14 binding directly to Mac-1.

In contrast, MRP-14 was found to bind to a distinct pertussis toxin-sensitive receptor on the neutrophil membrane, indicating that it interacts with neutrophils via a G protein-coupled receptor and that subsequent signalling leads to Mac-1 activation. As already described, FMLP, PAF and the chemokines mediate their effects through pertussis toxin-sensitive seven transmembrane spanning receptors which link to heterotrimeric G proteins (reviewed in (Murphy, 1994)). Two of the chemotactic S100 proteins, CP-10 and S100L, have also been shown to signal through pertussis toxin-sensitive receptors (Cornish et al., 1996; Komada et al., 1996); however, MRP-14 is unique among these proteins in that despite its receptor being G protein-coupled, it does not act as a chemoattractant. These differences suggests two possibilities: Firstly, that the signal transduction pathways associated with the G proteins coupled to its receptor differ from those associated with chemoattractant receptors which signal a larger array of functions; secondly, that the kinetics of receptor interaction or strength of signal might dictate the number of intracellular pathways activated.

Although, the exact signalling pathways for G protein-coupled receptor signalling have not been elucidated, many kinases have been identified as important for neutrophil

responses. For example, phosphatidylinositol 3-kinase has been implicated in Mac-1 adhesion to ligand (Knall et al., 1996; Metzner et al., 1997). In addition, phosphatidylinositol 3-kinase has also been shown to be necessary for superoxide production, degranulation and migration (Ahmed et al., 1995; Knall et al., 1997; Knall et al., 1996; Vlahos et al., 1995), but not for Mac-1 upregulation, Ca^{2+} mobilisation or actin polymerisation (Knall et al., 1997; Metzner et al., 1997; Vlahos et al., 1995). The sensitivity of MRP-14-induced Mac-1 activation and adhesion to inhibitors of PI 3-kinase suggests that MRP-14 signalling is at least partly the same as that of chemoattractants. The lack of superoxide production and degranulation in response to MRP-14, despite activation of PI 3-kinase can be resolved by the fact that MRP-14 does not stimulate intracellular Ca^{2+} mobilisation or PKC, which are necessary for these effector functions (Ahmed et al., 1995). The lack of migration in response to MRP-14 may be the result of no alteration of neutrophil cytoskeleton. Alternatively, chemotaxis in response to IL-8 was recently confirmed to be wortmannin- and LY294002-sensitive, but was not PD098059-sensitive (i.e. no MEK activation) (Knall et al., 1997). This suggests that the PI 3-kinase pathway must bifurcate before MEK activation and the fact that MRP-14 function is sensitive to MEK inhibition suggests that MRP-14 may only activate one of these pathways and not the pathway which leads to migration.

The steps preceding PI 3-kinase activation may involve the GTP-binding protein Ras. Indeed, non-adherent cells are rendered adherent by transfection with activated R-Ras, suggesting Ras may play a role in integrin activation (Zhang et al., 1996). Similar experiments with neutrophils would determine whether Ras is involved in MRP-14 activation of Mac-1. The fact that Ras can directly activate PI 3-kinase (Rodriguez-Viciana et al., 1994) suggests that the pathway of integrin activation involving PI 3-kinase may be initiated by Ras. Also the small GTP-binding protein Rho is directly involved in neutrophil adhesion in response to fMLP. Inhibition of Rho by the C3 exoenzyme failed to effect the respiratory burst and rise in $[Ca^{2+}]_i$ in response to fMLP, suggesting a more direct role in integrin-mediated adhesion (Laudanna et al., 1996). Therefore, it would also be interesting to examine the role of Rho in MRP-14 function.

The inability of PKC inhibitors to affect MRP-14 function provides some evidence for the lack of involvement of the alternate G protein signalling pathway involving phospholipase C (PLC) (reviewed in (Baggiolini et al., 1994)) in Mac-1 activation. Activation of PLC generates two second messengers, 1,4,5-inositoltrisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of Ca^{2+} from intracellular stores, resulting in a transient rise in $[Ca^{2+}]_i$, and DAG remains associated with the membrane

and activates PKC (Baggiolini et al., 1994). A rise in $[Ca^{2+}]_i$ is essential for the induction of exocytosis and the respiratory burst (see (Baggiolini et al., 1994)), but not Mac-1 upregulation (Jacobson and Schrier, 1993; Naccache et al., 1994), actin polymerisation (Sham et al., 1993) or migration (Jiang et al., 1997; Knall et al., 1997).

Calcium is also required for activation of phospholipase A_2 (PLA_2). PLA_2 has also been implicated in neutrophil adhesion (Jacobson and Schrier, 1993) as well as Mac-1 upregulation, cell spreading and degranulation (Jacobson and Schrier, 1993). A more recent report, however, found no evidence for PLA_2 in chemoattractant induced adhesion to fibrinogen (Metzner et al., 1997). This may be explained by the different PLA_2 inhibitors and/or ligands used in the two studies. Also, PLA_2 has been shown to be activated by MAP kinase (Lin et al., 1993), which may reflect an alternative signalling pathway to activation by calcium. Therefore, it may be interesting to test the role of PLA_2 in MRP-14-stimulated neutrophil adhesion. Tyrosine kinases have also been implicated in Mac-1-dependent neutrophil adhesion (Naccache et al., 1994) as well as many neutrophil responses, such as Mac-1 upregulation (Naccache et al., 1994), migration, and superoxide production (Yasui et al., 1994). The role of tyrosine kinases in MRP-14 function is unclear. Some activation of mAb 24 expression appears to be induced in unstimulated neutrophils (presumably by the separation and handling of neutrophils) which is inhibitable with the tyrosine kinase inhibitor herbimycin A. Further increases in mAb 24 expression in response to MRP-14 also appeared to be inhibited, however, it is unclear whether the effect was on the background tyrosine kinase activation. The inhibition of MRP-14-stimulated adhesion with herbimycin A supports the role of tyrosine kinase activation in MRP-14 function.

A greater knowledge of the signalling cascade generated by MRP-14 will come from measurements of kinase activity and phosphorylation of proteins identified in this study. Nevertheless, a model for MRP-14 function (and Mac-1 activation) can be constructed, based on the evidence presented and data described for chemoattractant responses discussed in this chapter (**Figure 5.12**). Briefly, MRP-14 binds to a G protein-coupled receptor and activates PI 3-kinase, which in turn activates Raf-1 or MEKK-1. Activation of either of these two results in the sequential activation of MEK-1 and MEK-2 and p42/44 MAP kinase. Activation of MAP kinase would then result in integrin activation.

It is likely that, in the context of an immune response, other factors could simultaneously activate neutrophils and generate a wider spectrum of neutrophil effector functions. In addition, ligation of Mac-1 by ligand or antibodies elicits intracellular signalling through the Mac-1 receptor itself (described as "outside in"

signalling), which lead to elevations in intracellular Ca^{2+}, superoxide production, upregulation of Mac-1 expression (Crockett-Torabi et al., 1995) and neutrophil spreading. Mac-1 adhesion to ligand also promotes activation of phospholipase D (Fållman et al., 1993) and tyrosine phosphorylation (Berton et al., 1994). In the context of endothelium, localised MRP-14 may provide an initial signal to neutrophils to activate their Mac-1 and firmly adhere to the vessel wall. Neutrophils accumulated here could then be directed into tissues by a chemotactic stimulus.

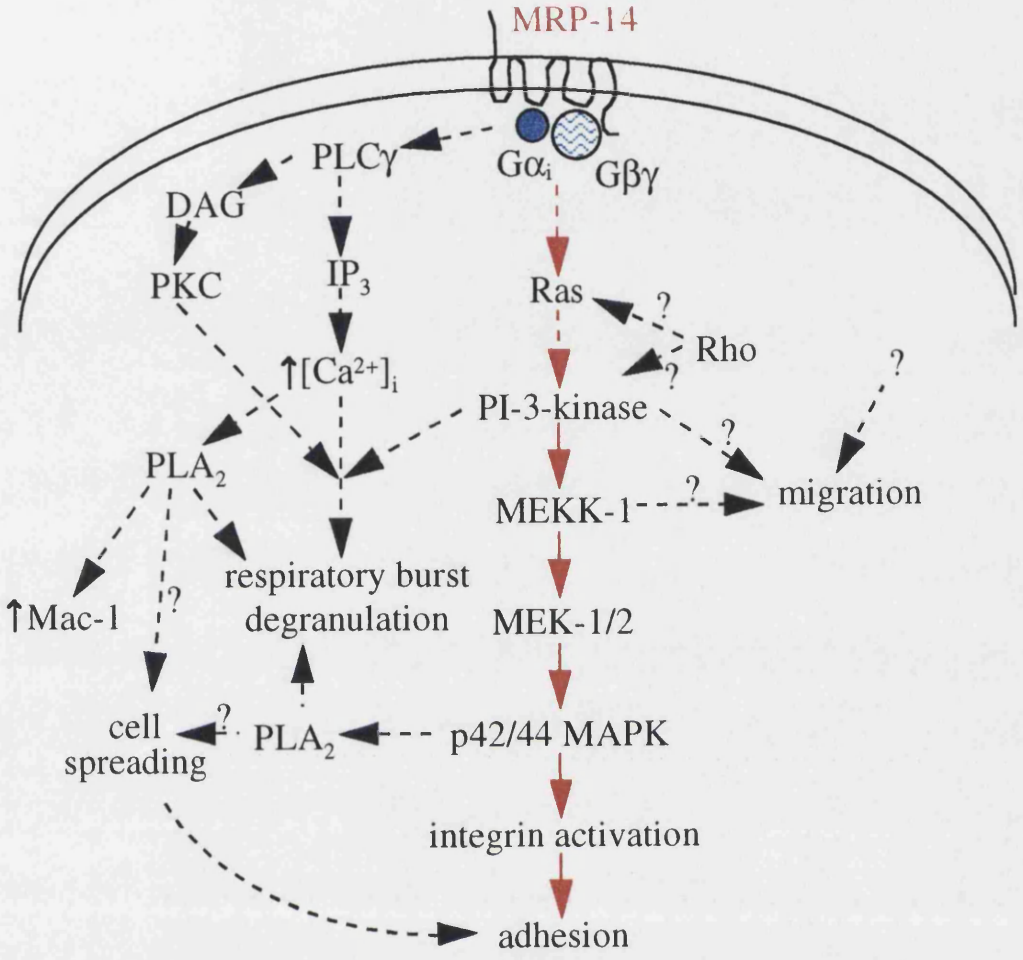

Figure 5.12: A model of neutrophil signal transduction in response to MRP-14. Schematic representation of the potential signal transduction pathways involved in the response to MRP-14 (and classical chemoattractants) via G protein-coupled receptors, leading to the activation of β_2 integrins and adhesion (red lines). Dotted lines represent a summary of data discussed describing the additional pathways activated by classical chemoattractants which result in other neutrophil effector functions. Question marks (?) indicate pathways as yet incompletely defined. The model is adapted from that of Avdi *et al.* (Avdi et al., 1996).

THE EFFECT OF MRP-14 ON T LYMPHOCYTES

6.1 INTRODUCTION

The transmigration of T lymphocytes from the blood to extravascular tissues is essential for successful immune surveillance and resolution of inflammatory (reviewed in (Springer, 1994)). The adhesive events necessary for leukocyte extravasation are well characterised; however the patterns of lymphocyte migration are more complex than those of myeloid cells. T lymphocytes not only participate in antigen-specific immune responses but also recirculate through lymphoid and non-lymphoid tissues. This is further complicated by the heterogeneity of the T lymphocyte population. For example, migration through specific endothelia is determined by whether the T cells express a naïve or memory phenotype (Mackay et al., 1990; Mackay et al., 1992). In spite of this complexity, the LFA-1 and VLA-4 integrins have been identified as important mediators of lymphocyte binding and transmigration, both *in vivo* and *in vitro* (Kavanaugh et al., 1991; Oppenheimer-Marks et al., 1991). As described previously, these adhesion molecules must be activated in order to facilitate transmigration and, as for the neutrophils, signalling through selectins and chemokine receptors has also been implied for T cell integrin activation. The evidence for selectin-mediated integrin activation comes from the induction of β_2 integrin-dependent adhesion of naïve lymphocytes to ICAM-1 by ligation of L-selectin with GlyCAM-1 (Hwang et al., 1996). *In vivo*, lymphocyte binding to high endothelial venules (HEV) is sensitive to pertussis toxin suggesting that in addition or alternate to integrin activation by selectins, G protein-coupled receptor signalling is involved (Bargatze and Butcher, 1993). The receptors on leukocytes for the chemokines and other chemoattractants are all G protein-coupled and a number of studies have addressed the question of whether chemokines can activate integrin adhesion as well as promoting migration. T lymphocytes preferentially recognise members of the CC chemokine family (reviewed in (Baggiolini et al., 1994)); however, the role of these chemokines in integrin activation has been controversial. Favouring such a role is the localised binding on endothelia of the CC chemokines, macrophage inflammatory protein (MIP)-1β (Tanaka et al., 1993)and monocyte chemotactic protein (MCP)-1 (Fuentes et al., 1995), placing them in an effective position for activating lymphocyte adhesion.

Indeed, MIP-1β is able to stimulate CD8+ T cell migration and adhesion to VCAM-1 (Tanaka et al., 1993). In agreement, another study also showed MIP-1β to stimulate activated CD8+ cell migration and adhesion to endothelium and, in addition, MIP-1α was shown to stimulate the same functions of activated CD4+ cells (Taub et al., 1993). This is contradictory to a similar study in which MIP-1β was shown to be chemotactic for CD4+ cells and not CD8+ (Schall et al., 1993). Despite MCP-1 localisation on the endothelium, MCP-1 promotes T cell adhesion to fibronectin but not to VCAM-1, ICAM-1 or endothelium (Carr et al., 1996), implying MCP-1 may play more of a role in adhesion of cells within the extracellular matrix. However, another study suggests that MCP-1, MIP-1α, MIP-1β, and RANTES are all able to induce T cell adhesion not only to fibronectin, collagen and laminin, but also to ICAM-1 and VCAM-1 (Lloyd et al., 1996). It is becoming obvious that the contradictions in these results may be explained by different activation states of the T lymphocytes, including the chemokine receptor expression levels (Mackay, 1996). One CXC chemokine, interferon-inducible protein (IP)-10, has been shown to preferentially stimulate the adhesion of activated T cells to endothelium (Taub et al., 1993), again suggesting that different activation states of T cells may dictate the adhesive response to chemokines. The neuropeptides, substance P and vasoactive intestinal peptide, both stimulate chemotaxis of unactivated T cells through their G protein-coupled receptors and stimulate T cell adhesion to ICAM-1 (substance P and VIP), VCAM-1 and fibronectin (VIP only) (Johnston et al., 1994; Vishwanath and Mukherjee, 1996).

All of the above studies have shown that either LFA-1, VLA-4 or VLA-5 mediates the T cell adhesion to ligands ICAM-1, VCAM-1 and fibronectin in response to chemoattractants. There are two different mechanisms of activation of β_1 and β_2 integrins on T cells. Firstly, activation of the T cell through T cell receptor cross-linking, or phorbol ester stimulation, induces LFA-1 and β_1 integrin adhesion through avidity changes in the integrin accompanied by other cellular events such as cell spreading and $[Ca^{2+}]_i$ (Porter and Hogg, 1997; Stewart et al., 1996). Another mechanism of activation of β_1 and β_2 integrins on T cells is by direct binding to the integrins by activating mAbs or divalent cations, such as Mg^{2+} or Mn^{2+} (Dransfield et al., 1992; Faull and Ginsberg, 1995; Jakubowski et al., 1995; Porter and Hogg, 1997). Unlike other activators, these agents bind directly to the integrin and elicit a conformational change, which is thougth to enable the integrin to bind to ligand with higher affinity.

6.2 RESULTS

The function of Mac-1 on T cells is relatively unknown as T cells do not normally express Mac-1. Both CD4+ and CD8+ T cells have been shown to express Mac-1 only under conditions of antigenic stimulation (Chapman et al., 1996; Dianzani et al., 1989; Forsyth and Mathews, 1996; McFarland et al., 1992; Nielsen et al., 1994). T cells were, therefore, thought to be a good model to investigate the effect of MRP-14 on LFA-1 in the absence of Mac-1. In addition, unstimulated neutrophils express very low levels of β_1 integrins (Reinhardt et al., 1997), so T cells are also a good model for investigating the effect of MRP-14 on α_4 and $\alpha_5\beta_1$ integrin adhesion.

6.2.1 THE PHENOTYPE OF T LYMPHOCYTES CULTURED IN THE PRESENCE OF IL-2

The T cells used in this study were derived from PBMCs stimulated with phytohaemagglutinin (PHA) for 4 days, followed by incubation with IL-2 for 10 days (Dransfield et al., 1992). All experiments were performed using cells which had been in culture for days 8-14. Phenotypically, cells from day 10 were all CD3+ indicating a homogeneous population of T lymphocytes (**Figure 6.1A**). Of the CD3+ cells, approximately 1/3 were CD4+ and 2/3 CD8+ (**Figure 6.1B**). All CD3+ cells were also CD45RO positive (**Figure 6.1C**), suggesting the cultured T cells were expressing a memory phenotype.

6.2.2 THE EFFECT OF MRP-14 ON T LYMPHOCYTE ADHESION TO IMMOBILISED ICAM-1

As LFA-1 is the predominant β_2 integrin on the T cell surface (see **Figure 6.2A**), it was of interest to test whether MRP-14 could induce T cell adhesion to ICAM-1Fc via LFA-1. MRP-14 titrated from 0-2μM induced adhesion to ICAM-1Fc. Adhesion was not saturated even at 2μM MRP-14 and only ~20% adhesion was induced (**Figure 6.3A**). This is compared with ~60% T cell binding stimulated with the β_2 activating mAb KIM 185 or ~35% with Mn^{2+} ions (**Figures 6.3A and 6.4**). Antibody blocking of ICAM-1Fc binding revealed that MRP-14-stimulated binding was fully inhibited with the Mac-1 mAb (2LPM19c) and not the LFA-1 mAb (MEM25), indicating that MRP-14 activates Mac-1, but not LFA-1, adhesion (**Figure 6.3B**). In contrast, binding induced by the β_2 activating mAb KIM185 and Mn^{2+} were fully inhibited by the LFA-1 mAb and not the Mac-1 mAb (**Figure 6.4** and data not

shown). The small amount of inhibition of MRP-14 induced binding to ICAM-1 seen with the LFA-1 mAb (**Figure 6.3B**) was not consistent and appeared to represent the blocking of homotypic aggregation seen in all wells (data not shown).

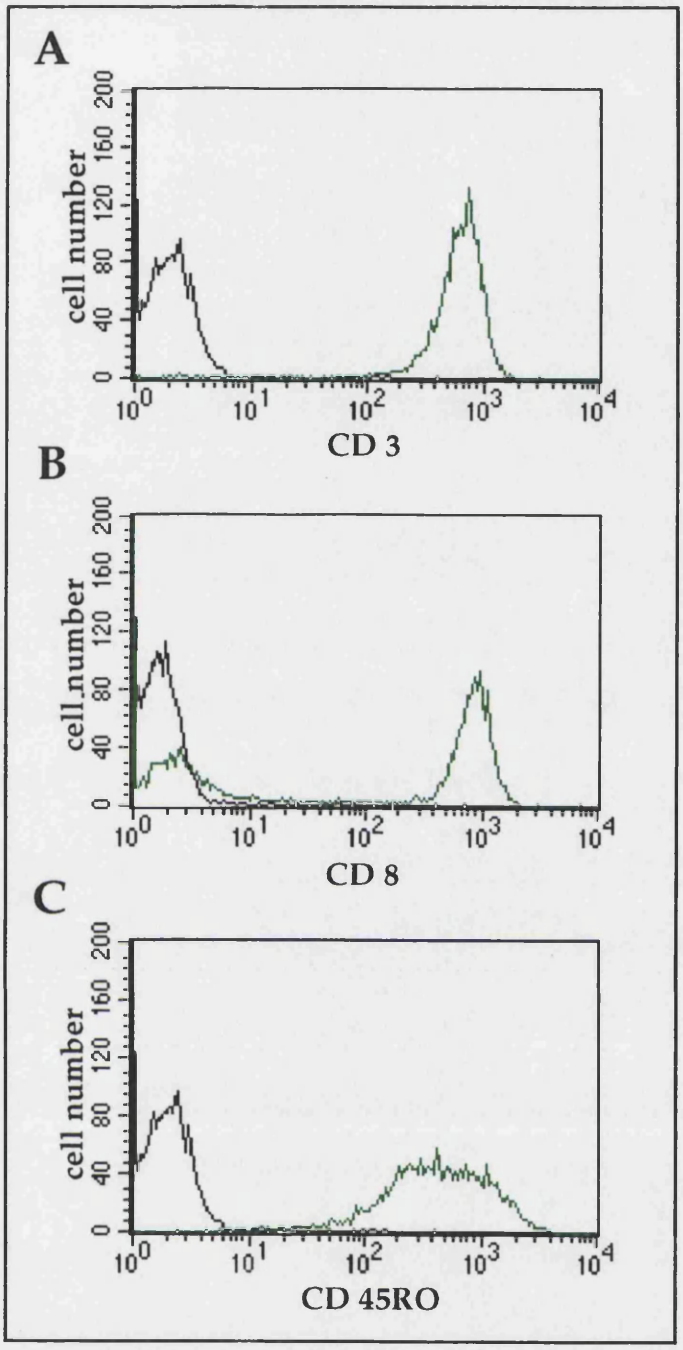

Figure 6.1: Phenotypic analysis of cultured T lymphocytes. Peripheral blood mononuclear cells (PBMC) were stimulated with phytohaemagglutinin (PHA) and cultured in the presence of interleukin (IL)-2. After 10 days in culture, flow cytometric analysis of cultured cells using monoclonal antibodies (see Materials and Methods) showed cells were **A.** 100% CD3 positive; **B.** 65% CD8 positive and; **C.** 100% CD45RO positive. Antibody binding was detected using FITC-conjugated goat anti-mouse immunoglobulins. Secondary antibody alone is represented as (———) and cells incubated with primary and secondary antibodies as (———).

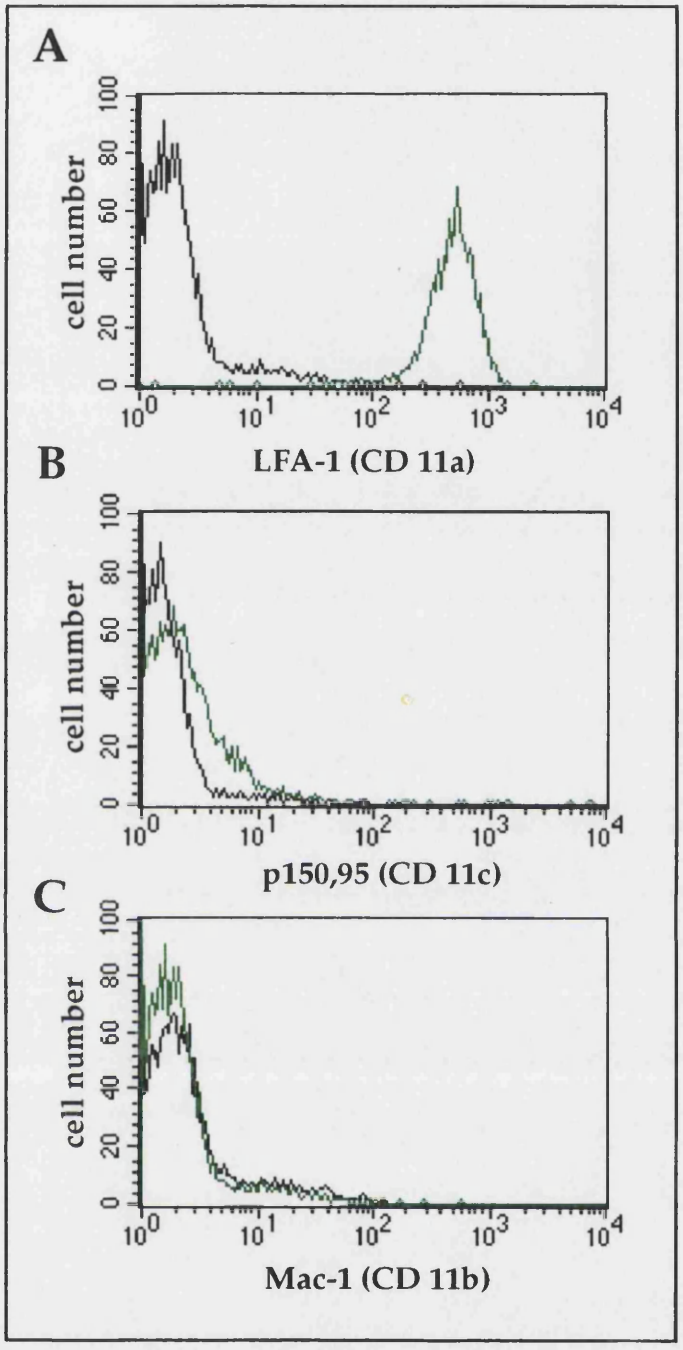

Figure 6.2: Expression of β_2 integrins on cultured T lymphocytes. Cultured T cells A. abundantly express LFA-1 (CD11a), **B.** show minimal expression of p150,95 (CD11c), and **C.** are negative for Mac-1 (CD11b) expression. Antibody binding was detected using FITC-conjugated goat anti-mouse immunoglobulins. Secondary antibody alone is represented as (———) and cells incubated with primary and secondary antibodies as (———).

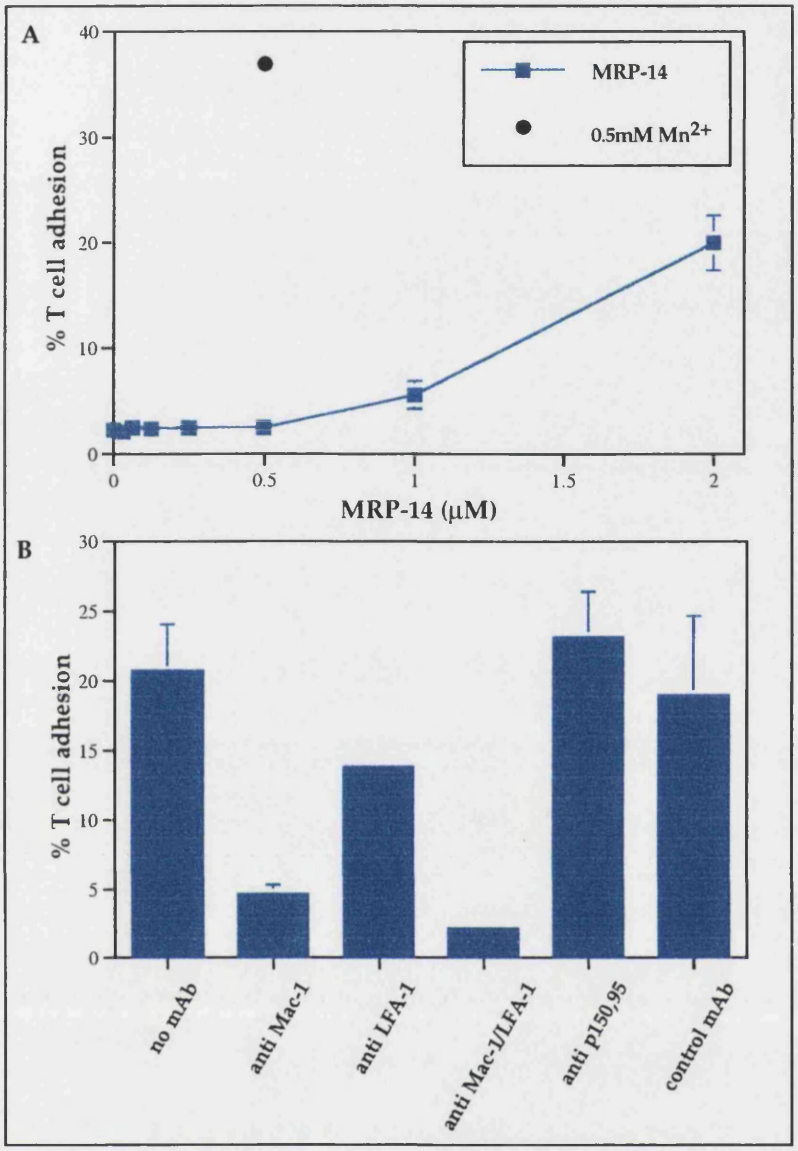

Figure 6.3: The effect of recombinant MRP-14 on T cell adhesion to ICAM-1Fc. A. MRP-14, titrated from 0-2μM, induces T cell adhesion to ICAM-1Fc, in a dose-dependent manner, with maximum adhesion of 20% at 2μM (—■—). In comparison, Mn^{2+} (0.5mM) induces ~35% T cell adhesion (●). **B.** MRP-14 (2μM) -induced T cell adhesion to ICAM-1 is inhibited by the Mac-1 blocking mAb, 2H3, and not by antibodies to LFA-1 or p150,95 or an isotype (IgG1) control antibody. Results are expressed as mean % T cell adhesion ± standard deviation. Data presented are from one experiment representative of six. Antibodies were used at concentrations indicated in the Materials and Methods.

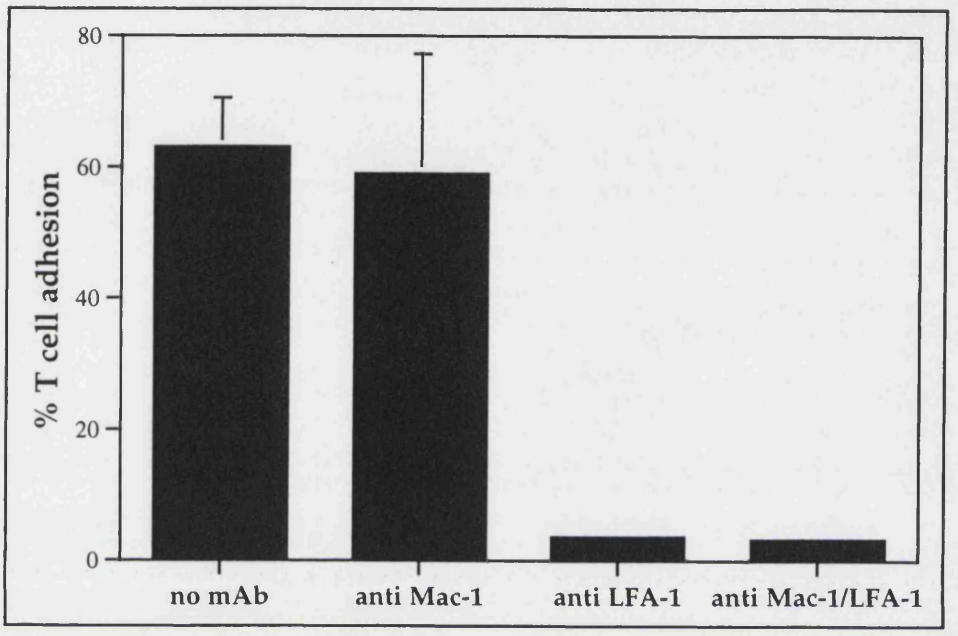

Figure 6.4: The effect of antibodies on T cell ICAM-1 adhesion induced by the β_2 integrin activating mAb, KIM185. KIM185 stimulates ~60% adhesion to ICAM-1Fc, which is completely LFA-1-dependent as shown by the ability of anti-LFA-1 mAb, MEM25, to fully inhibit all binding. The anti-Mac-1 mAb, 2H3, and an isotype control mAb, had no effect on T cell adhesion to ICAM-1. Results are expressed as the mean % T cell adhesion ± standard deviation. The data shown are from one experiment representative of six. Antibodies were used at concentrations indicated in the Materials and Methods.

6.2.3 THE EFFECT OF MRP-14 ON T CELL ADHESION MOLECULE EXPRESSION

On the T cells used in this study, β_2 integrin expression was due to high levels of LFA-1 (CD11a) (**Figure 6.2A**), with small amounts of p150,95 expression (**Figure 6.2B**), and no expression of Mac-1 (CD11b) (**Figure 6.2C**). The ability of MRP-14 to induce significant T cell adhesion to ICAM-1 via Mac-1 was difficult to explain, based on the lack of expression of this β_2 integrin on unstimulated T cells (**Figure 6.2C and 6.5A**). Therefore, the possibility that MRP-14 might induce the expression of Mac-1 on T cells was investigated. Under the conditions of the adhesion assay, Mac-1 expression was determined in the presence and absence of MRP-14. In agreement with the adhesion results, MRP-14 (1μM) induced the expression of Mac-1 on the T cells (**Figure 6.5A**). As a control, Mn^{2+} ions (0.5mM) did not induce an increase in Mac-1 expression (**Figure 6.5B**). Increases in Mac-1 expression in the presence of MRP-14 were also seen with three other non-blocking Mac-1 mAbs (data not shown). This increase in Mac-1 expression was temperature-dependent, with no expression being induced at 0°C, suggesting the increased expression was energy dependent (**Figure 6.5C**). In addition, 1μM MRP-14 was able to induce an increase in the expression of p150,95 on the T cells (**Figure 6.5D**). This increased expression was also confirmed by three other p150,95 mAbs (data not shown). Other adhesion molecules were similarly tested for their increased expression on the surface of T cells in response to 1μM MRP-14. In contrast to Mac-1 and p150,95, LFA-1 ($\alpha_L\beta_2$, **Figure 6.6A**), VLA-4 ($\alpha_4\beta_1$, **Figure 6.6B**), VLA-5 ($\alpha_5\beta_1$, **Figure 6.6C**) and L-selectin (**Figure 6.6D**) remained unchanged from their unstimulated expression levels in the presence of MRP-14.

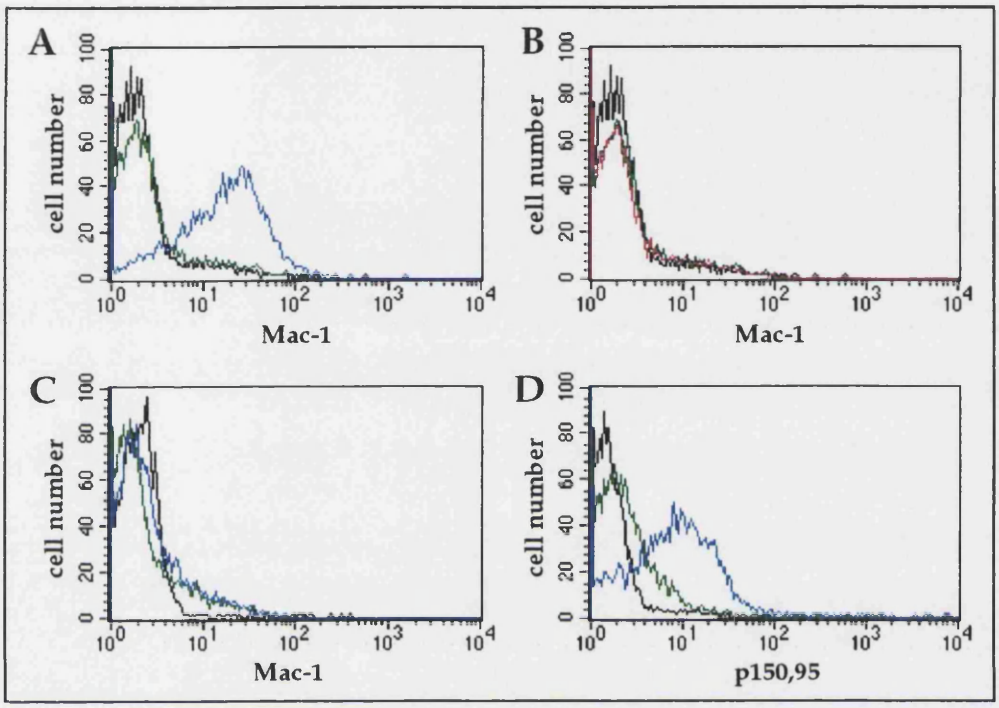

Figure 6.5: MRP-14 stimulates increased Mac-1 expression on cultured T cells. A. MRP-14 at 1μM (———) stimulated Mac-1 expression on cultured T cells after 30 min at 37°C. Unstimulated cells (———) do not express Mac-1 as their FL-1 profile shows no increase above unstained cells (———) **B.** Unlike MRP-14, Mn^{2+} (———) does not stimulate Mac-1 expression on T cells. **C.** MRP-14-induce Mac-1 expression (———) is temperature-dependent, with no increase being detected at 0°C. **D.** MRP-14 also increases the expression of p150,95 on T cells. Unstimulated and unstained cells are indicated as in A. One representative experiment of three is shown.

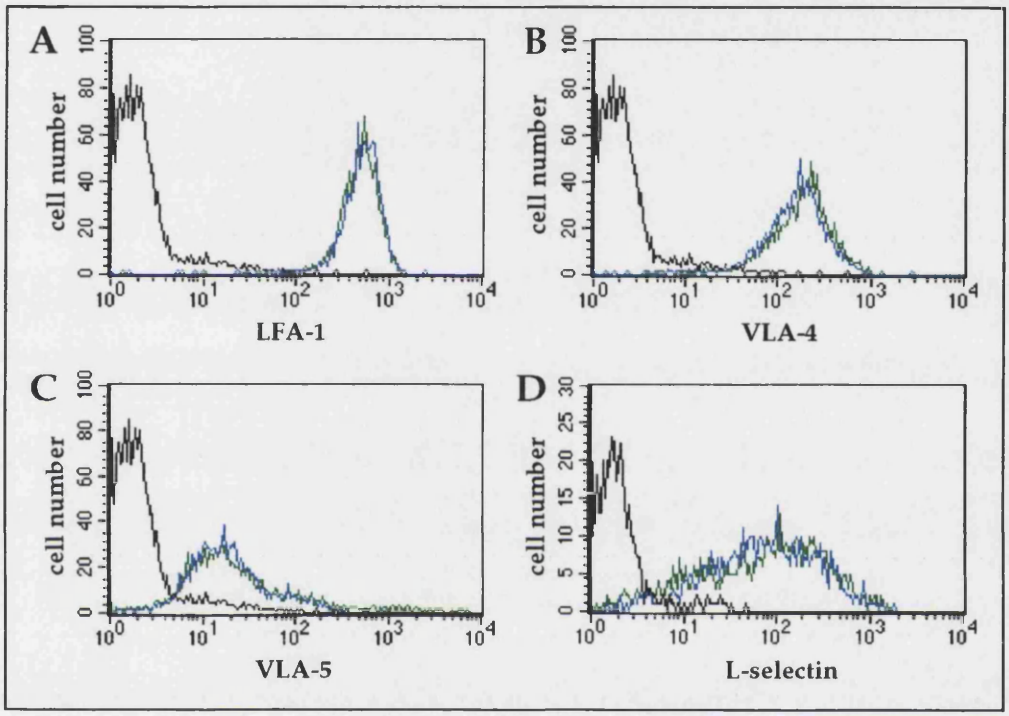

Figure 6.6: The effect of MRP-14 on T cell expression of other adhesion molecules. MRP-14 showed no effect on T cell expression of **A.** LFA-1, **B.** VLA-4, **C.** VLA-5, and **D.** L-selectin. Primary antibodies were used at concentrations indicated in the Materials and Methods. Antibody binding was detected by the FITC-conjugated goat anti-mouse immunoglobulins (Sigma). Secondary antibody alone is represented as (———). Unstimulated and MRP-14-stimulated cells are indicated as (———) and (———), respectively. Data shown are from a single experiment, representative of six.

6.2.4 EXPRESSION OF THE MAb 24 ACTIVATION REPORTER EPITOPE ON T LYMPHOCYTES STIMULATED WITH MRP-14

It was still unclear as to whether the adhesion induced by MRP-14 was brought about purely by the increased expression of Mac-1, perhaps in a highly avid or clustered form or whether, as for the neutrophils (see chapter 4), MRP-14 could also alter β_2 integrin affinity. To test this hypothesis, the expression of the β_2 integrin activation reporter epitope, recognised by mAb 24, was evaluated. MRP-14 at 1μM induced the expression of the mAb 24 epitope (**Figure 6.7A and C**), although to a lesser extent than that induced by Mn^{2+} (**Figure 6.7B and D**). As expected, the β_2 integrin activation epitope was expressed by Mac-1, and not LFA-1, in response to MRP-14 as seen by the ability of the Mac-1 blocking mAb to completely inhibit the binding of mAb24 (**Figure 6.7A**). MAb 24 expression on Mn^{2+}-stimulated T cells was unaffected by the Mac-1 mAb, confirming that Mac-1 is not activated by Mn^{2+} (**Figure 6.7B**). In addition, the induction of the mAb 24 epitope by MRP-14 is dependent on metabolic energy, as the replacement of glucose in the buffer system with 2-deoxyglucosamine and sodium azide is inhibitory to mAb 24 epitope expression (**Figure 6.7C**). In contrast, Mn^{2+}-stimulated mAb 24 expression is unaffected by inhibiting metabolic energy (**Figure 6.7D**), which is in agreement with the fact that Mn^{2+} regulates β_2 integrin function by directly binding to them.

6.2.5 THE BINDING OF SOLUBLE ICAM-1 TO T LYMPHOCYTES

The ability of MRP-14 to solely stimulate, Mac-1 and not LFA-1, is further supported by the binding of soluble ICAM-1. MRP-14, at 1μM, induces soluble ICAM-1Fc (sICAM-1) binding at all concentrations tested, although binding was not saturated at 100 μg/ml (~450nM) (**Figure 6.8A**). Mn^{2+}, at 0.5mM, induced approximately 50% of the binding seen in the presence of MRP-14, but again binding was not saturated at 100μg/ml (**Figure 6.8B**). Using mAbs to Mac-1 and LFA-1, the specificity of sICAM-1 binding to T cells stimulated with either 1μM MRP-14 or 0.5mM Mn^{2+} was determined. All sICAM-1 binding in response to MRP-14 was inhibited by the Mac-1 mAb 2H3, but unaffected by the LFA-1 mAb 38 (**Figure 6.8A**). In contrast, Mn^{2+}-stimulated sICAM-1 binding was fully inhibited by the LFA-1 mAb and unaffected by the Mac-1 mAb (**Figure 6.8B**). The ability of MRP-14 to stimulate soluble ICAM-1 binding to Mac-1 is further evidence that MRP-14 induces the high affinity state of this integrin.

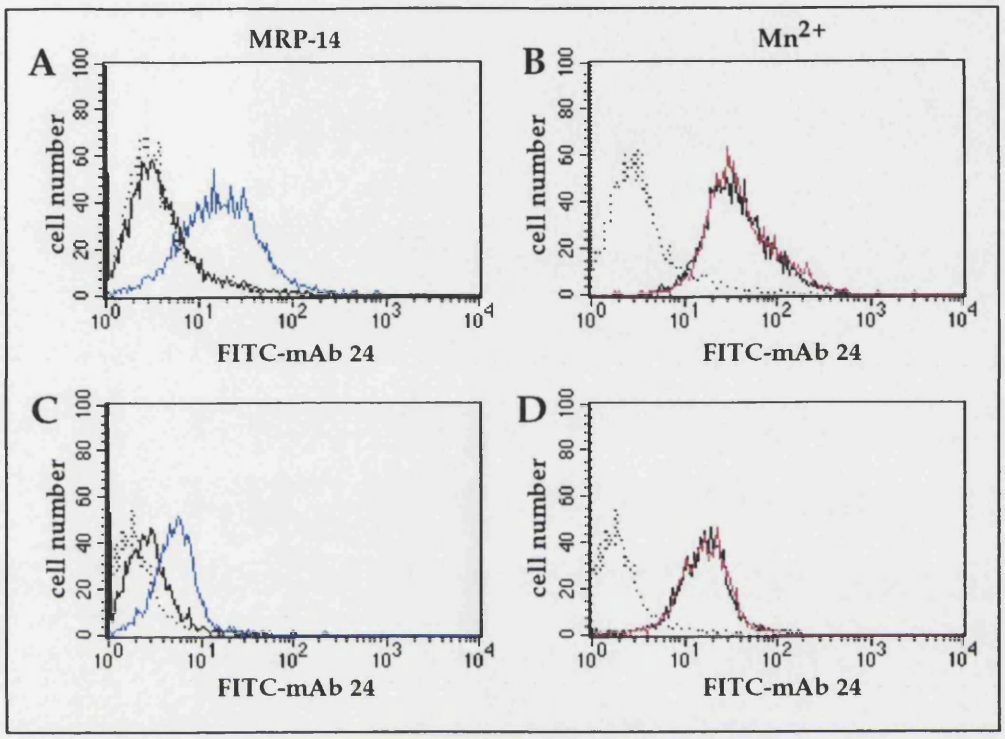

Figure 6.7: The effect of MRP-14 on the expression of the β_2 integrin activation epitope, recognised by mAb 24. A. MRP-14 (1μM) (———) and **B.** Mn^{2+} (0.5mM) (———) activate the expression of the β2 integrin activation epitope, recognised by mAb 24. FITC-conjugated mAb 24 was used at 10μg/ml. Control FITC-conjugated mAb staining is indicated as (------). MRP-14-induced mAb 24 expression is inhibited with the Mac-1 blocking mAb, 2H3; whereas, Mn^{2+}-stimulated mAb 24 expression was unaffected (———). MAb **24 expression** induced by **C.** MRP-14, but not **D.** Mn^{2+}, is dependent on metabolic energy as mAb 24 expression is inhibited by the substitution of sodium azide and 2-deoxyglucosamine in the buffer. Key as for A. and B., where inhibitor is represented by (———).

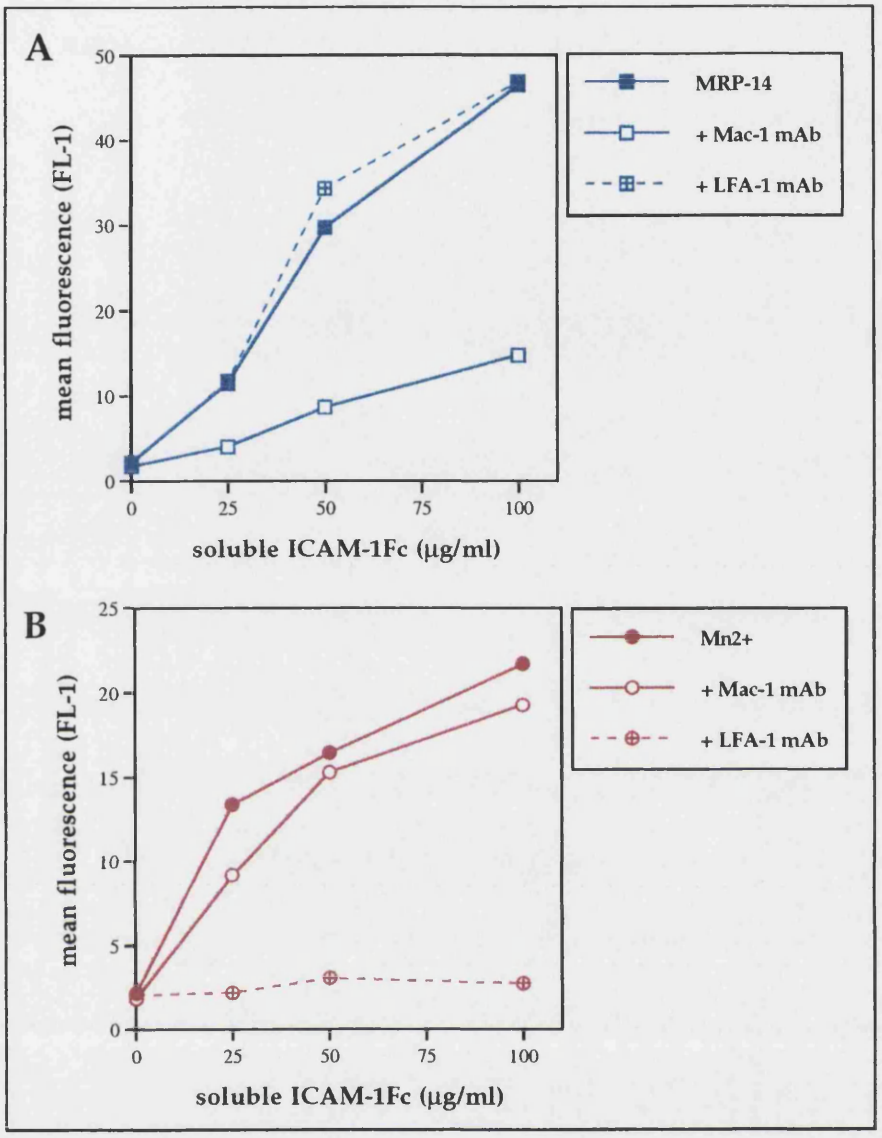

Figure 6.8: Soluble ICAM-1Fc binding to T cells. A. MRP-14 (2µM) induces soluble ICAM-1Fc binding to T cells (—■—) This binding was inhibited by the anti-Mac-1 mAb 2H3 (—□—) and not by the LFA-1 mAb 38 (—⊞—). **B.** Mn^{2+} (0.5mM) also stimulates soluble ICAM-1Fc binding (—●—), but, in contrast to MRP-14, all binding was inhibited by the anti-LFA-1 mAb (—⊕—) and not by the anti-Mac-1 mAb (—○—). Soluble ICAM-1Fc binding was detected by 10µg/ml goat anti-human Fc specific antibody (Jackson Immunoresearch Laboratories). Other mAbs were used at concentrations indicated in the Materials and Methods. Data shown are from one representative experiment of two.

6.2.6 THE EFFECT OF MRP-14 AND MRP-8 ON T LYMPHOCYTE ADHESION TO IMMOBILISED FIBRINOGEN

Experiments based on those for neutrophil adhesion were performed to test the ability of MRP-14 to induce T cell adhesion to fibrinogen. Consistent with the induction of Mac-1 expression, MRP-14, titrated over the same range as for neutrophils (0-2 μM), was able to induce T cell adhesion to fibrinogen. Similar to the neutrophils, T cell adhesion was maximal at 1μM with ~40% of the total cells added adhering (**Figure 6.9A**). All T cell binding induced by MRP-14 was inhibited by the Mac-1 (CD11b) mAb 2LPM19c (**Figure 6.9A**), confirming that Mac-1 was mediating the adhesion observed. The specificity of the MRP-14 function was again confirmed by the ability of MRP-8 to inhibit the adhesion induced by 1μM MRP-14, with 50% inhibition again occurring at ~1:1 ratio of MRP-8/MRP-14 (**Figure 6.9B**). Other T cell stimulants known to activate LFA-1 adhesion to ICAM-1, such as divalent cations (e.g. Mn^{2+}), activating mAbs, and phorbol esters (see (Stewart and Hogg, 1996)), were tested for their ability to induce T cell Mac-1 adhesion to fibrinogen. Neither Mn^{2+} nor any other stimulant tested induced T cell adhesion to fibrinogen (data not shown), however, under the same conditions all were able to induce LFA-1 adhesion to ICAM-1 (**Figures 6.3A and 6.4** and data not shown).

6.2.7 SOLUBLE FIBRINOGEN BINDING TO T CELLS STIMULATED WITH MRP-14

From the above results, it was apparent that MRP-14 was able to induce both an increase in Mac-1 expression on the T cells and an increase in Mac-1 function, evident by increased T cell adhesion to ICAM-1 and fibrinogen (**Figure 6.3, 6.5A and 6.9**). MAb 24 epitope expression on T cell Mac-1 (**Figure 6.7A**) suggested that the increased Mac-1 function was due to an increase in affinity, which was confirmed by the ability of T cells to bind soluble ICAM-1 in response to MRP-14 (**Figure 6.8A**). Further proof that MRP-14 directly alters the affinity of Mac-1 was provided by measuring T cell binding of soluble fibrinogen. Unstimulated T cells failed to bind FITC-fibrinogen even at 500nM (**Figure 6.10A**). In contrast, in the presence of 1μM MRP-14, T cells were induced to bind FITC-fibrinogen, reaching a plateau between 160nM and 300nM and then increasing FITC-fibrinogen binding again with increasing concentration to 500nM (**Figure 6.10A**). The saturation at 160nM is 16 fold greater fibrinogen than the amount required for saturation of MRP-14-stimulated neutrophils. The specificity of binding was again confirmed by the ability of unlabelled

fibrinogen and the blocking Mac-1 mAb 2LPM19c to inhibit all soluble fibrinogen binding (**Figure 6.10B**). As expected, Mn^{2+} did not induce any T cell soluble fibrinogen binding (data not shown).

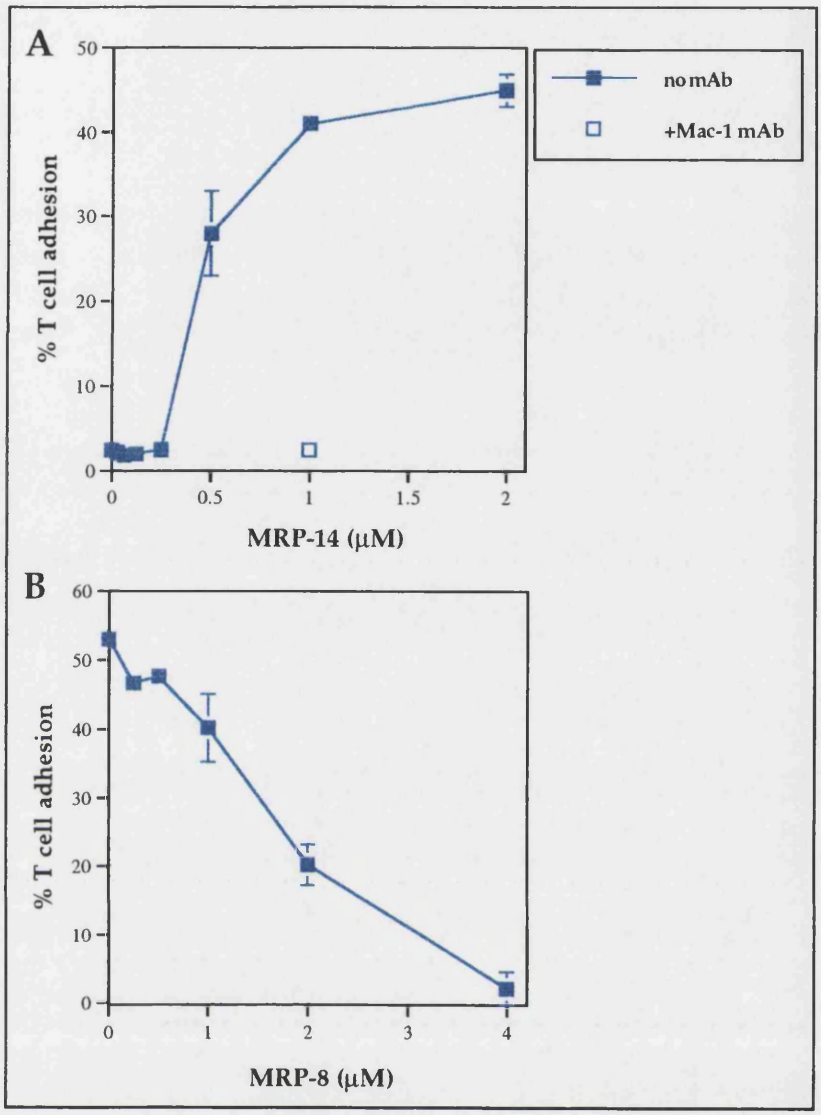

Figure 6.9: The effect of MRP-14 on T cell adhesion to fibrinogen. A. MRP-14 was titrated from 0-2 μM for the ability to induce T cell adhesion to fibrinogen (—■—). Maximum adhesion of ~40% was elicited with 1μM MRP-14. T cell adhesion in response to 1μM MRP-14 was fully inhibited by 20μg/ml Mac-1 mAb 2LPM19c (□). Results are expressed as the mean % of T cell adhesion ± standard deviation. The data shown are from one experiment representative of six. **B.** Recombinant monomeric MRP-8, titrated from 0-4 μM, inhibits the adhesion to fibrinogen induced by 1μM recombinant MRP-14. Results are expressed as the mean % of T cell adhesion ± standard deviation. The data shown are from one experiment representative of three.

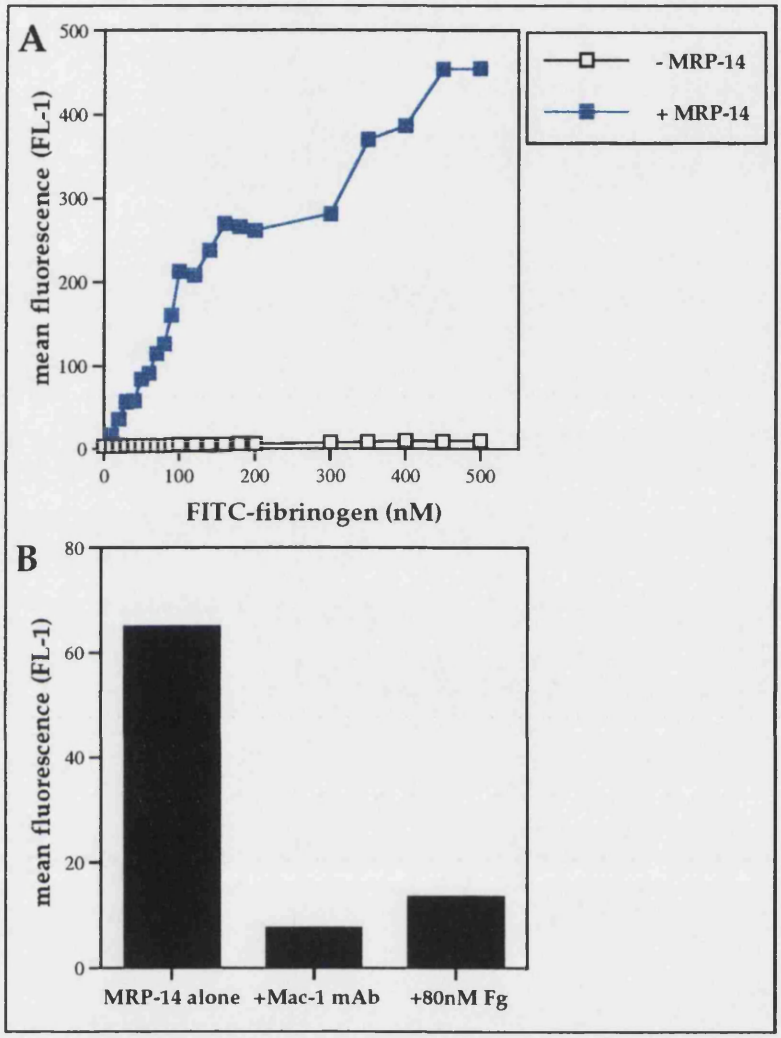

Figure 6.10: The effect of MRP-14 on the binding of soluble fibrinogen to T lymphocyte Mac-1. A. MRP-14 (—■—) but not unstimulated (—□—) T cells are induced to bind FITC-fibrinogen (n=6). **B.** The specificity of binding was demonstrated by the complete inhibition of 20nM bound FITC-fibrinogen by the addition of the Mac-1 blocking mAb 2LPM19c or 80nM unlabelled fibrinogen.

6.2.8 DOES MRP-14 STIMULATE ADHESION VIA OTHER T CELL INTEGRINS?

Unlike neutrophils, which do not express β_1 integrins, cultured T cells express varying levels of all β_1 integrins. Of particular relevance are the α_4 and α_5 β_1 integrins, which are expressed at high levels and play roles in lymphocyte migration (Roth et al., 1995). Their expression levels on cultured T cells are shown in Figure 6.6B and C. The α_4 integrins bind to VCAM-1 and both α_4 and α_5 β_1 integrins bind to fibronectin. Using immobilised fibronectin and VCAM-1 assays, the ability of MRP-14 to induce α_4- and/or α_5 -mediated adhesion, therefore, were tested. MRP-14, at 1μM, induced ~30% binding of T cells to fibronectin (**Figure 6.11**). This was in comparison to the 45% binding in response to treatment with 0.5mM Mn^{2+} ions (**Figure 6.11**). Some background binding was observed in the presence of MRP-14 to the blocking agent gelatin. This binding was <10% of the total cells and inhibited with a Mac-1 blocking mAb, 2LPM19c (**Figure 6.11** and data not shown). The effect of mAbs to the β_1 and β_2 integrins revealed that all of the MRP-14-stimulated binding was $\alpha_5\beta_1$ dependent as seen by the complete inhibition using either an α_5 (SAM-1) or a β_1 (P5D2) mAb. A small amount of adhesion was blocked with either the Mac-1 or α_4 (HP1/2) blocking mAbs, consistent with the amount of blocking seen with the isotype-matched control mAb (**Figure 6.11**). Similarly, 0.5mM Mn^{2+}-induced binding was inhibited by mAbs to α_5 and β_1 with no significant contribution of $\alpha_4\beta_1$. binding. In contrast to MRP-14, Mn^{2+} did not stimulate any background binding to gelatin and binding to fibronectin was unaffected by the Mac-1 blocking mAb (**Figure 6.11**). The lack of $\alpha_4\beta_1$ function in response to MRP-14 was confirmed by the binding of T cells to VCAM-1. High background levels of T cell adhesion were often seen to VCAM-1, which were attributable to $\alpha_4\beta_1$; however MRP-14 was not able to stimulate any α_4 adhesion above this level (data not shown). This confirms that MRP-14 selectively activates T cell $\alpha_5\beta_1$ adhesion to fibronectin, as well as Mac-1 adhesion to fibrinogen and ICAM-1.

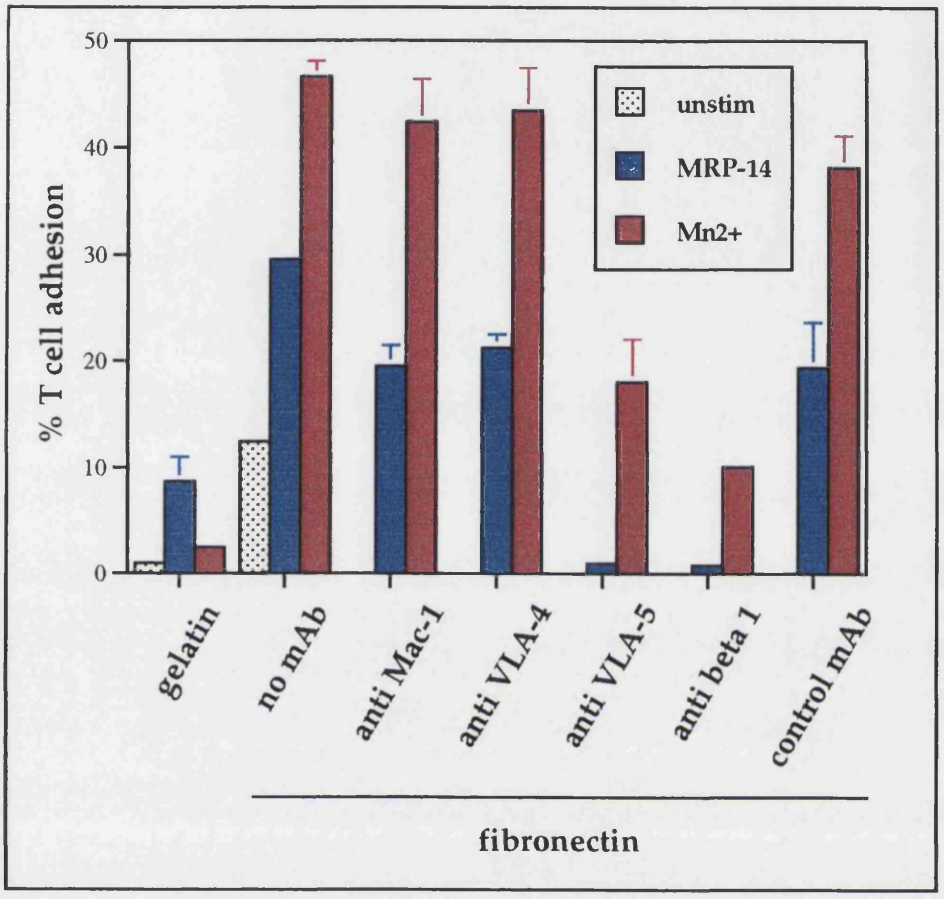

Figure 6.11: The effect of MRP-14 on T cell adhesion to fibronectin. MRP-14 was tested for the ability to induce T cell adhesion to fibronectin. MRP-14 at 1μM induced adhesion of ~30% total T cells added (■), compared to ~45% induced by 0.5mM Mn^{2+} (■). T cell adhesion in response to 1μM MRP-14 and Mn^{2+} were both inhibited by mAbs to either α_5 or β_1 integrin subunits. Unstimulated T cell binding is indicated as (□). Results are expressed as the mean % of T cell adhesion ± standard deviation. The data shown are from one experiment representative of six.

6.2.9 MRP-14 BINDS DIRECTLY TO T LYMPHOCYTES

As seen for neutrophils (**Figure 5.1**), MRP-14 functions by directly binding to the T cell membrane. Binding of FITC-conjugated MRP-14 to T cells at 0°C was maximal at 2-4 μM and was dependent on divalent cations (**Figure 6.12A** and data not shown). Specificity of binding was determined by the ability of unlabelled MRP-14 to inhibit the binding of 2μM FITC-MRP-14 (**Figure 6.12B**). The next step was to investigate the nature of the MRP-14 receptor on the T cells. Two conclusions can be made from the results presented so far. Firstly, that MRP-14 is unlikely to be binding to Mac-1 itself, based on the results in Figure 5.3 and the fact that MRP-14 still binds to the T cells at 0°C (**Figure 6.12A**), where no induction of Mac-1 expression is observed (**Figure 6.5C**). Secondly, the induction of mAb 24 expression and the binding of soluble fibrinogen by MRP-14 is dependent on metabolic energy (**Figure 6.8C** and data not shown), suggesting intracellular signalling is responsible for MRP-14 Mac-1 activation on T cells.

Preliminary experiments, to investigate the receptor for MRP-14, however, have been inconclusive. MRP-14-induced adhesion to fibrinogen is not affected by pertussis toxin, as for the neutrophils (**Figure 5.4** and data not shown); however, mAb 24 expression induced by MRP-14 was inhibited by pertussis toxin (data not shown). PI-3-kinase and MAP kinase inhibitors, which blocked the MRP-14-stimulated neutrophil mAb 24 expression and adhesion to fibrinogen (**Figures 5.6, 5.8, 5.10**), were also ineffective in blocking T cell adhesion or mAb 24 expression (data not shown). Consistent with the neutrophil results is the inability of the PKC inhibitor (*bis*-indolylmaleimide) to affect T cell adhesion or mAb 24 expression (data not shown). The nature of the signals generated by MRP-14 in T cells, therefore, needs to be further investigation. Current experiments in the laboratory are being performed to identify the MRP-14 receptor on T cells.

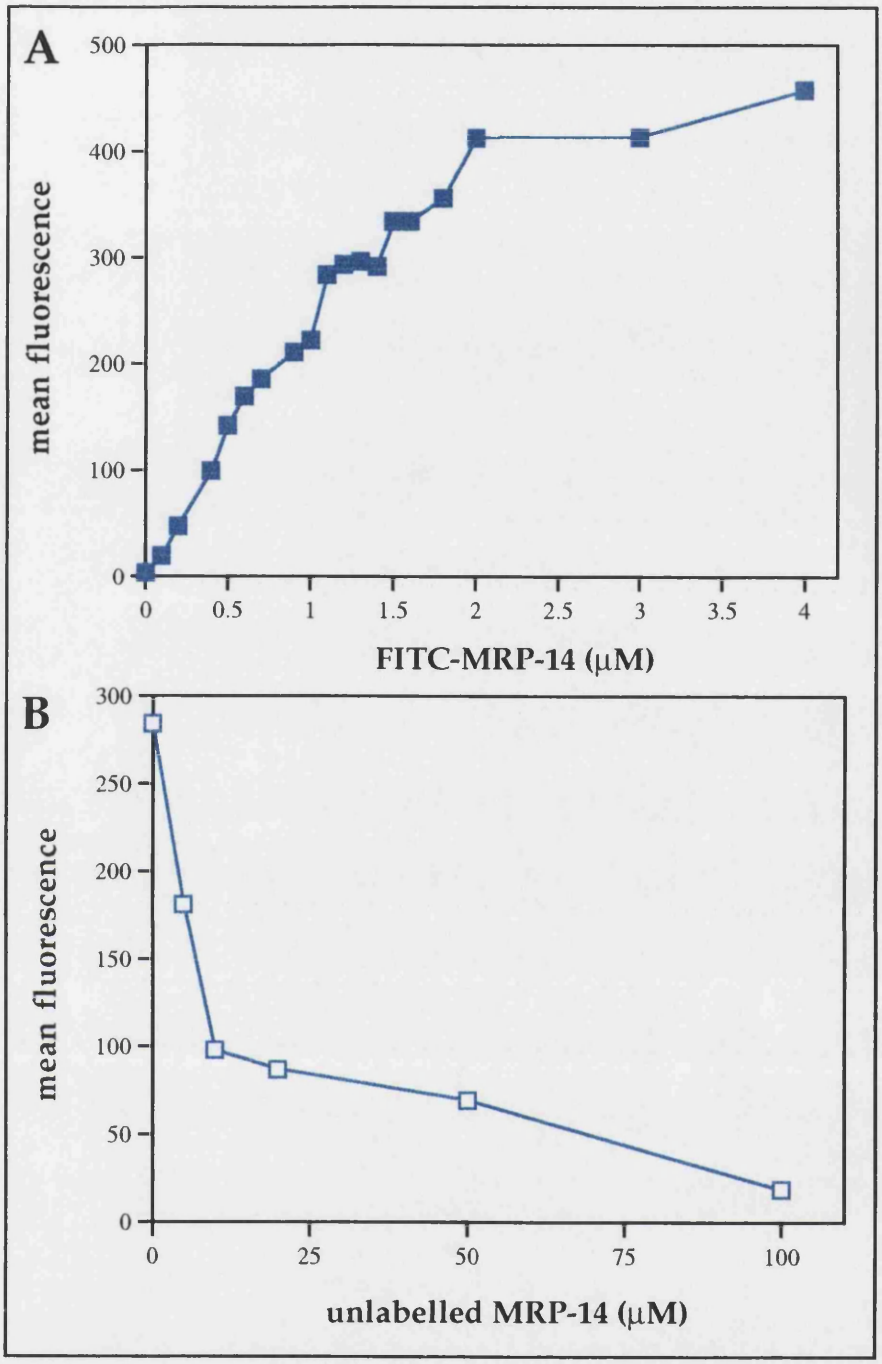

Figure 6.12: MRP-14 also binds directly to T cells. A. FITC-MRP-14, titrated from 0-4 μM, binds to the T cell membrane in a dose-dependent manner, saturating at 2μM. **B.** This saturating concentration (2μM) of FITC-MRP-14 is fully inhibited by co-incubation with unlabelled MRP14, titrated from 0-100 μM. Data are from a single experiment representative of six.

6.3 DISCUSSION

The results presented in this chapter represent the first demonstration of Mac-1 expression by T cells after stimulation with a physiological protein, other than antigen. In contrast to myeloid cells, the function of Mac-1 on T cells is relatively unknown. Only a few reports of Mac-1 expression on T cells have been published; however, all reports conclude that Mac-1 is a marker for CD8+ antigen-primed cells, as well as memory cells (Dianzani et al., 1989; Forsyth and Mathews, 1996; McFarland et al., 1992; Nielsen et al., 1994). Of these reports, only two describe functions for Mac-1 on T cells (Forsyth and Mathews, 1996; Nielsen et al., 1994). In one report, virally-infected mice were treated with an antibody to Mac-1 which inhibited the extravasation of CD8+ cells to sites of inflammation, implying that Mac-1 on T cells plays a role in migration to sites of inflammation (Nielsen, et al., 1994). In the second report, Mac-1 was found to be the adhesion receptor for *Candida albicans*, again suggesting that Mac-1 is involved in the cytotoxic T cell response to microorganisms (Forsyth and Mathews, 1996). The ability of MRP-14 to stimulate Mac-1 adhesion to ICAM-1 and fibrinogen is only, therefore, the third report of Mac-1 function on T cells.

Unlike "inside out" signalling, through T cell receptor cross-linking, phorbol ester or chemokine stimulation, MRP-14 does not activate LFA-1 adhesion to ICAM-1. Instead MRP-14 function is restricted to activating the newly expressed Mac-1. In addition, this Mac-1 activation is through changes in Mac-1 affinity, evident by the induction of soluble fibrinogen and ICAM-1 binding. T cell receptor cross-linking and phorbol ester stimulation of LFA-1 on T cells occurs through changes in avidity of the integrin and not changes in affinity; no soluble ligand binding is induced and adhesion is facilitated by LFA-1 clustering and changes in the cytoskeleton (Burn et al., 1988; Peter and O'Toole, 1995; Stewart et al., 1996). Neutrophil Mac-1 adhesion can also be stimulated through changes in avidity brought about by clustering of Mac-1 on the membrane and changes in the cytoskeleton (Detmers et al., 1987). There is no evidence for such avidity changes in response to MRP-14, as adhesion to fibrinogen was not inhibited by cytochalasin D (data not shown). Therefore, MRP-14 activates high affinity Mac-1 on T cells. Although, high affinity Mac-1 has been demonstrated for on myeloid cells activated with factors, such as ADP ((Altieri and Edgington, 1988; Altieri et al., 1990) and Chapter 3), no such evidence for Mac-1 on T cells exists. MRP-14 is the only factor described to date to activate Mac-1 expression and high affinity Mac-1 adhesion.

The temperature dependence of the MRP-14 induction of Mac-1 expression indicates that the increased expression is an active process, confirmed by the inability of Mn^{2+},

which binds directly to integrins, to similarly increase Mac-1 expression. The fact that this increased expression occurs within 30 minutes, suggests that Mac-1 (and p150,95) must be preformed within the cell. No evidence exists for T cells as to the intracellular localisation of Mac-1 and p150,95, however, it could be speculated that some forms of storage vesicles exist which are mobilised to the membrane upon stimulation.

The ability of MRP-14 to selectively activate Mac-1 and $\alpha_5\beta_1$ and not LFA-1 and $\alpha_4\beta_1$ is interesting since out these 4 integrins, $\alpha_5\beta_1$ and Mac-1 are the only ones to be recycled within cells (Bretscher, 1992). As these four integrins are all present on the same T cell, these results imply that LFA-1 and $\alpha_4\beta_1$ integrins must be regulated differently to Mac-1 and $\alpha_5\beta_1$. No evidence for affinity regulation of LFA-1 by physiological proteins exists, therefore, it may be possible that proteins, such as chemokines, regulate LFA-1 by changes in avidity. The $\alpha_4\beta_1$ integrins are known to function differently from other integrins as it can bind endothelium under flow conditions and mediate T cell rolling and arrest (Berlin et al., 1995), suggesting that they are already in a higher affinity state. Recent results from our laboratory have also identified that a hierarchy exists within T cell integrins, with the activation of LFA-1 inhibiting the function of β_1 integrins (Porter and Hogg, 1997). This may be relevant in the context of the sequence of events required for successful transmigration.

The ability of MRP-14 to bind to the surface of the T cell in the absence of Mac-1 expression, suggests that MRP-14 is not activating high Mac-1 affinity by binding directly to it, but is operating through another receptor. The induction of mAb 24 expression and soluble ligand binding by MRP-14 is dependent on metabolic energy of the T cells, also suggesting MRP-14 operates through a specific receptor. This is further supported by the simultaneous activation of T cell binding to fibronectin by the $\alpha_5\beta_1$ integrin. The sensitivity of neutrophil MRP-14-induced mAb 24 expression to pertussis toxin suggests that the receptor for MRP-14 is G protein-coupled. However, the inability of pertussis toxin to inhibit MRP-14-induced T cell adhesion to fibrinogen is contradictory. One explanation could be that the pertussis toxin is itself activating Mac-1 adhesion. Pertussis toxin has previously been shown to activate PKC and calcium transients in T cells (Rosoff et al., 1987) by binding to a specific cell surface receptor (Rogers et al., 1990). This is corroborated by the T cell spreading of cells adherent to fibrinogen observed in the presence of pertussis toxin (data not shown). The inhibition of MRP-14 induced mAb 24 expression on neutrophils may, therefore, be a true indication of the nature of the MRP-14 receptor. Experiments looking at GTPase activity in T cells stimulated with MRP-14 will provide more definitive proof

that MRP-14 is operating through a G protein-coupled receptor. Downstream signals from the putative MRP-14 receptor have not yet been identified and it appears, from the results so far, that in T cells, unlike neutrophils, there is no PI 3-kinase or MAP kinase activity stimulated, although a more extensive investigation would be needed to prove this. The inconsistency in results between neutrophils and T cells may reflect the incompatibility of the assay systems between cell types. Alternatively, it may reflect differences in signalling generated by MRP-14 in the different cells, either by the use of different receptors or the coupling of different signalling pathways to the same receptor. One indication that differences exist between T cells and neutrophils is the ability of MRP-14 to cause the upregulation of Mac-1 and p150,95 in T cells, but not in neutrophils. Future experiments in the laboratory will address both these possibilities.

DISCUSSION

The aim of this thesis was to investigate the role of the human S100 family Ca^{2+}-binding proteins, MRP-8 and MRP-14, in leukocyte adhesion. This investigation was prompted by the observations in Figure 1.7, where extracellular MRP-8/14 was detected, by monoclonal antibody staining, deposited onto the surface of endothelium. Using model systems of *in vitro* leukocyte adhesion, extracellular functions for MRP-8 and MRP-14 have been described for the first time. The results presented in this thesis demonstrate that MRP-14 can stimulate neutrophil and T lymphocyte adhesion via the β_2 integrin, Mac-1. As well, MRP-14 is able to promote T lymphocyte adhesion to fibronectin, which, in the cells used, was dependent on $\alpha_5\beta_1$ integrins. In contrast, the other β_2 integrin, LFA-1, abundantly expressed on these cells, is not stimulated to bind ligand. This suggests that MRP-14 is selective in its ability to activate integrin family members. It is interesting to note that both Mac-1 and $\alpha_5\beta_1$ integrins can be recycled within these cells, whereas LFA-1 cannot.

MRP-14 was unique among the S100 family members in stimulating adhesion, as no other S100 protein tested, including the heterodimeric partner of MRP-14, MRP-8, was able to stimulate adhesion. In fact, MRP-8 has been identified, in this study, as a natural and specific inhibitor of MRP-14 function. The inhibition by MRP-8 of the adhesion induced by MRP-14 has been demonstrated to be via the formation of the MRP-8/14 heterodimer, confirming the lack of ability of the native MRP-8/14 heterodimer to induce adhesion.

7.1 POTENTIAL SOURCES OF EXTRACELLULAR MRP-14 *IN VIVO*

Since the heterodimeric complex represents the major form of MRP-8 and MRP-14 in the myeloid cytosol, it must be assumed that in order to be functional, in the extracellular environment, MRP-14 must be either dissociated from the complex or expressed alone, and secreted. Recent evidence suggests that MRP-14 may be secreted as a monomer from stimulated monocytes. This is supported by the findings that MRP-14 has been detected as a monomer in the serum of patients with cystic fibrosis at higher levels than that of MRP-8.

Much information on MRP-8/14, published in recent years, has centred around the translocation of MRP-8/14, within the cell, from the cytosol to the cell membrane in association with intermediate filaments. Based on these results and the recent publication, describing secretion of MRP-/14 from monocytes, it would appear that the intracellular MRP-8/14 complex may be functionally distinct from that secreted into the extracellular environment. The intracellular heterodimer is recruited to the membrane as a phosphorylated protein complex after stimulation of the cells with agonists known to generate increases in $[Ca^{2+}]_i$. This implies that the phosphorylated form of the protein may be important in a Ca^{2+}-dependent intracellular signal transduction pathway. The secreted form of the MRP-8/14 protein complex need not necessarily be phosphorylated, but does depend on the activation of PKC within the cells. In contrast, phosphorylation and translocation of MRP-8/14 cannot be stimulated by activation of PKC.

7.2 MRP-14 IS A UNIQUE ACTIVATOR OF LEUKOCYTE ADHESION

MRP-14 affects the adhesive state of Mac-1 on neutrophils and T cells by directly increasing the affinity of Mac-1, as demonstrated by soluble ligand binding and the expression of the β_2 integrin activation reporter epitope, recognised by mAb 24. In both cell types, no alteration of the cytoskeleton was observed and the protein was not able to induce cell migration. This contrasts MRP-14 with three other S100 proteins, CP-10, S100L and psoriasin, known to function as specific leukocyte chemoattractants. In neutrophils, MRP-14 function is very selective as other effector functions of the neutrophil, such as $[Ca^{2+}]_i$ flux, superoxide production, or exocytosis are not stimulated by the protein. Together, these findings suggest that MRP-14 is unique in its ability to selectively activate Mac-1-mediated adhesion by increasing the affinity of the integrin for ligand. To date, only stimuli known to bind directly to Mac-1, such as divalent cations or activating mAbs, can generate high affinity status of the integrin. Other stimuli which operate through specific cell surface receptors, such as chemokines, generate increased integrin function (although not necessarily increased affinity) in conjunction with the activation of cell effector functions, such as migration. This implies that MRP-14 may be functioning by direct binding to Mac-1; however, the evidence presented in this thesis suggests that MRP-14 has a distinct cell surface receptor.

7.3 EVIDENCE FOR A SPECIFIC MRP-14 RECEPTOR

There are several lines of evidence to suggest that MRP-14 does not bind to Mac-1 but instead binds to a distinct receptor. Firstly, MRP-14 binds directly to neutrophils, T lymphocytes and an erythroleukaemic cell line (K562). Under the conditions of the assay, both the T cells and the parent K562 cells were negative for expression of Mac-1. Secondly, MRP-14 binding to cells was not inhibited by antibodies to Mac-1 directed to the 'T' domain ligand binding site or the C-terminus, where other proteins, such as HMWK, bind to Mac-1. Thirdly, on neutrophils, the ability of MRP-14 to stimulate Mac-1 mediated adhesion and activation of this β_2 integrin is inhibited by pertussis toxin. This strongly suggests that MRP-14 is operating via a pertussis toxin-sensitive G protein-coupled receptor. Fourthly, evidence is presented in this thesis to support an intracellular signal transduction pathway. In neutrophils, the ability of MRP-14 to induce expression of the β_2 integrin activation reporter epitope and to stimulate Mac-1-mediated adhesion is dependent on activation of PI 3-kinase, and the MAP kinase cascade, but not PKC. For T cells, although the signal transduction pathway has not been defined, MRP-14 appears to be induce exocytosis in these cells as determined by the induction of Mac-1 and p150,95 expression, which was both temperature- and energy-dependent. Furthermore, in T cells, MRP-14 is able to stimulate the activation of another subfamily of integrins, the β_1 integrins.

7.4 CONCLUSIONS

The results presented in this thesis are important for two reasons:

1) This is the first demonstration of a function for extracellular MRP-8/14, identifying specific functions for both MRP-8 and MRP-14 in the regulation of leukocyte adhesion.

2) MRP-14 has been identified as a unique activator of integrin affinity and may prove useful in defining the exact intracellular pathway leading to integrin activation.

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APPENDIX I

PBS-A	g/L
NaCl	80
KCl	2.5
Na_2HPO_4	14.3
KH_2PO_4	2.5

RPMI-1640 (ICRF)	g/L
RPMI powder	103.9
$NaHCO_3$	37
Penicillin	0.6
Streptomycin	1

RPMI-1640 (Sigma)	g/L
$Ca(NO_3)_2.4H_2O$	0.1
$MgSO_4$	0.049
KCl	0.4
$NaHCO_3$	2.0
NaCl	6.0
Na_2HPO_4	0.8
D-glucose	2.0
L-glutamine	0.3

DMEM (Sigma)	g/L
$CaCl_2.2H_2O$	0.265
$MgSO_4$	0.098
KCl	0.4
$NaHCO_3$	3.7
NaCl	6.4
NaH_2PO_4	0.109
D-glucose	4.5
L-glutamine	0.584

HBSS (GIBCO BRL)	g/L
Kcl	4.0
KH_2PO_4	0.6
NaCl	80
$Na_2HPO_4.7H_2O$	0.90
D-glucose	10.0

PUBLICATIONS ARISING FROM THIS WORK

The following references are publications which arose from the work described in this thesis. They are located in the pocket inside the back cover.

Signaling mechanisms and the activation of leukocyte integrins.
R. A. Newton, M. Thiel and N. Hogg
Journal of Leukocyte Biology. 1997. 61: 422-426.

The human S100 protein MRP-14 is a novel activator of the β2 integrin Mac-1 on neutrophils.
R. A. Newton and N. Hogg
Journal of Immunology. 1998. (In press)

The human S100 protein MRP-14 activates Mac-1 adhesion of T lymphocytes through increased expression and affinity of Mac-1.
R. A. Newton and N. Hogg
Manuscript in preparation.