Regulation of Macrophage Inflammatory Protein-1α Expression by Haemopoietic Growth Factors

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Declaration

I declare that all work presented in this thesis was performed by me personally unless acknowledged otherwise.

Dedication

This thesis is dedicated to my family.
In memory of Beatrice and Stanley Jarmin, and Hilda Skinner.

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ABSTRACT

Macrophage inflammatory protein-1 alpha (MIP-1α) is a pro-inflammatory cytokine and a regulator of haemopoiesis. Little is currently known regarding the network of regulatory factors controlling the expression of MIP-1α, within the bone marrow microenvironment. This study was therefore initiated to further understand how expression of this gene is regulated by haemopoietic growth factors. Macrophages are likely to be an important source of MIP-1α within the bone marrow and this study has therefore used primary murine bone marrow-derived macrophages and Northern blot analysis to examine the expression of MIP-1α following treatment with various growth factors.

The results presented in this thesis show that treatment of bone marrow-derived macrophages with granulocyte-macrophage colony-stimulating factor (GM-CSF) results in a striking increase in MIP-1α mRNA levels, relative to control cells. This increase in MIP-1α mRNA expression was also followed by an increase in MIP-1α protein levels in these cells, suggesting that the potent stimulatory effect of GM-CSF on macrophage-derived MIP-1α expression may be of physiological or pathological significance. Since GM-CSF, interleukin- (IL-) 3 and IL-5 all share a common β-chain subunit in their receptors and can functionally substitute for each other in certain cell types, primary macrophages were also treated with IL-3 or IL-5. A similar increase in mRNA levels was found when macrophages were treated with IL-3, but not IL-5. An increase in the expression of mRNAs for three other CC chemokines: MIP-1β, JE and MARC, was also found following treatment with GM-CSF or IL-3, implying that the potent stimulatory effects of GM-CSF and IL-3 are not restricted to MIP-1α and may therefore also have an impact on the expression of other CC chemokines.

Previous work from our group has provided evidence of an endogenous reciprocal relationship in macrophages, between MIP-1α and transforming growth factor-beta (TGF-β), in which TGF-β acts to suppress MIP-1α expression. To investigate the potential effect of GM-CSF and IL-3 on the relationship between TGF-β and MIP-1α, the effect of TGF-β on the GM-CSF-or IL-3-induced expression of MIP-1α mRNA was assessed. TGF-β was able to suppress the GM-CSF-or IL-3-

induced expression of MIP-1α mRNA, but this suppression was not complete. These results suggest that GM-CSF and IL-3 may therefore be two potential stimulatory signals, that may be sufficiently potent to overcome the TGF-β mediated block on MIP-1α expression. Similar effects on the expression of the GM-CSF or IL-3 induced expression of MIP-1β, JE and MARC were also observed. The significance of this is unclear, but may reflect the widely recognised function of TGF-β as an immuno-suppressive molecule.

A previous study in our laboratory suggested that TGF-β reversibly downregulated the expression of MIP-1α receptors on the murine FDCPmix cell line. Therefore, the effect of GM-CSF and IL-3 on the expression of various receptors for MIP-1α on bone marrow-derived macrophages was also examined and an increase in the expression of CCR1 mRNA levels was observed, but no change was seen in the level of mRNA for CCR5. This suggests that CCR1 and CCR5 expression may be differently regulated in bone marrow-derived macrophages. TGF-β was observed to have only a minimal suppressive effect on the expression of CCR1 and CCR5 mRNA. Binding studies revealed that treatment of bone marrow macrophages with GM-CSF also resulted in an increase in the overall levels of MIP-1α receptors on these cells. Scatchard analysis showed that GM-CSF increased the number of MIP-1α receptors with no change in the affinity of the remaining receptors for MIP-1α ligand. The K_d's for the receptors detected in this study are similar to those previously reported for murine CCR1, but different to those for CCR5. This observation, together with the lack of effect of GM-CSF on CCR5 expression suggests, that this increase in MIP-1α receptor numbers probably reflects an increase in the cell surface expression of CCR1. To investigate the functional significance of this increase in MIP-1α receptors, the effect of GM-CSF on the ability of macrophages to mobilise calcium in response to MIP-1α was examined. In one experiment, treatment of bone marrow-derived macrophages with GM-CSF appeared to result in a slight increase in the ability of these cells to respond to MIP-1α. In most cases however, their ability to flux was highly variable, suggesting they were not an ideal choice for calcium flux analysis and therefore the pro-monocytic human THP-1 cell line was used instead. GM-CSF stimulation of THP-1 cells resulted in an increase in the expression of CCR1 mRNA, similar to that observed in bone marrow-derived macrophages, but no increase in

calcium mobilisation was observed in GM-CSF treated THP-1 cells in response to MIP-1α. The functional significance of the observed GM-CSF-induced increase in CCR1 expression therefore remains to be determined.

Using RT-PCR analysis, a preliminary investigation of MIP-1α expression in tissues derived from a GM-CSF transgenic mouse, revealed that an increased expression of MIP-1α occurred in samples from the eye (and perhaps also the bone marrow) of a GM-CSF transgenic mouse. Although preliminary, these results suggest that increased expression of MIP-1α may occur under circumstances in which elevated GM-CSF expression occurs.

The data presented in this thesis suggest that expression of GM-CSF or IL-3 may be one mechanism through which the levels of MIP-1α and other β chemokines produced within the bone marrow by macrophages is increased. Indeed, the observation that TGF-β was only partially able to inhibit the induction of MIP-1α by GM-CSF and IL-3, implies that GM-CSF and IL-3 may be sufficiently potent to overcome the proposed TGF-β mediated block on MIP-1α expression. Although it is impossible to say with certainty, whether these observations reflect an involvement in stem cell proliferative control or in inflammatory responses, these results clearly have implications for both. In summary, the results presented in this thesis suggest that the expression of MIP-1α and other chemokines in bone marrow macrophages, is regulated through the combined interaction of the positive stimuli exerted by GM-CSF and IL-3 and the negative stimulus of TGF-β.

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ABBREVIATIONS

^{32}P	^{32}Phosphorous
5-FU	5-fluorouracil
7TMsGPCR	7 transmembrane spanning G protein-coupled receptor
Ab	antibody
Ag	antigen
AIDS	Acquired immune deficiency syndrome
AML	acute myelogenous leukaemia
Ara-C	cytosine arabinoside
ATP	adenosine triphosphate
βc	common β chain receptor subunit
β_{IL-3}	IL-3 receptor β chain
BFU	burst-forming unit
BM	bone marrow
BMM	bone marrow-derived macrophages
BSA	bovine serum albumin
C	cysteine
cDNA	complementary deoxyribonucleic acid
CFC	colony-forming cell
CFU-A	colony-forming unit arbitrary
CFU-E	colony-forming unit erythroid
CFU-GM	colony-forming unit granulocyte-macrophage
CFU-S	colony-forming unit spleen
CM	conditioned medium
CML	chronic myeloid leukaemia
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSF	colony-stimulating factor
CTP	cytidine triphosphate
d	day (e.g. day 8)
DARC	Duffy antigen receptor for chemokines
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine triphosphate
DHS	donor horse serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphates
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Epo	erythropoietin

ES	embryonic stem
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GM	granulocyte-macrophage
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
HCl	hydrochloric acid
HEK	human embryonic kidney
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HPC	haemopoietic progenitor cell
HPP-CFC	high proliferative potential colony-forming cell
HSC	haemopoietic stem cell
HU	hydroxyurea
IFN	interferon
Ig	immunoglobulin (e.g. IgE)
IL-	interleukin (e.g. IL-3)
IP10	interferon-inducible protein-10
IP3	inositol triphosphate
KSHV	kaposi's sarcoma-associated herpesvirus
LB	Luria broth
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LTRC	long-term repopulating cell
LTC-IC	long-term culture intitiating cell
LTBMC	long-term bone marrow culture
M-CSF	macrophage colony-stimulating factor
MCP	monocyte chemotactic protein
MEM	minimal essential medium
Mig	monokine induced by interferon-γ
MIP	macrophage inflammatory protein
MOPS	3-(N-morpholino) propanesulphonic acid
MPIF	myeloid progenitor inhibitory factor
mRNA	messenger ribonucleic acid
MRP	macrophage inflammatory protein related protein
MS	multiple sclerosis
m.w.	molecular weight
NK	natural killer cell
OD	optical density
op	osteopetrotic
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PF-4	platelet factor four

PKC	protein kinase C
PLC	phospholipase C
PTX	perussis toxin
r	recombinant
R	receptor
RA	rheumatoid arthritis
RANTES	regulated on activation normal T-cell expressed and secreted
Rh	receptor homologue
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SCF	stem cell factor
SCI	stem cell inhibitor
SDF	stromal derived factor
SDS	sodium dodecyl sulphate
Sl	steel
SSC	standard saline citrate
TEMED	tetramethylenediamine
tg	transgenic
TGF	transforming growth factor
T_h	T helper
TNF	tumour necrosis factor
TPO	thrombopoietin
Tris	tris(hydroxymethyl)aminomethane
U	units
UTP	uridine triphosphate
UV	ultraviolet
v/v	volume for volume
w/v	weight for volume
wt	wild type

PART I: INTRODUCTION

CHAPTER 1: The Haemopoietic System

1.1 The Haemopoietic System: An Overview

The haemopoietic system is essential for the maintained viability of an individual. It can be considered as a highly complex cellular system that enables the derivation of mature immuno-haemopoietic cells, through the processes of proliferation, commitment and differentiation. There are at least eight mature blood cell types, functionally distinct from each other, but which are ultimately derived from a single self-renewing pluripotent stem cell that differentiates via various different committed progenitor cells (some of which are summarised in figure 1.1). This complex system has evolved to cope with the on-going production of the vast numbers of cells needed on a daily basis, simply to maintain the stasis of the mature immuno-haemopoietic system. In fact it is estimated that as many as 3.7×10^{11} haemopoietic cells are needed per day to maintain human homeostasis. In addition to this homeostatic role, the haemopoietic system must also be able to respond rapidly with a dramatic increase in cell numbers, in order to be able to combat infection and disease (inducible haemopoiesis). The many processes involved in regulating the haemopoietic system are likely to be complex and elaborate and yet they must also be remarkably efficient because despite the enormous scope for abnormalities, diseases such as leukaemia and anaemia are relatively rare.

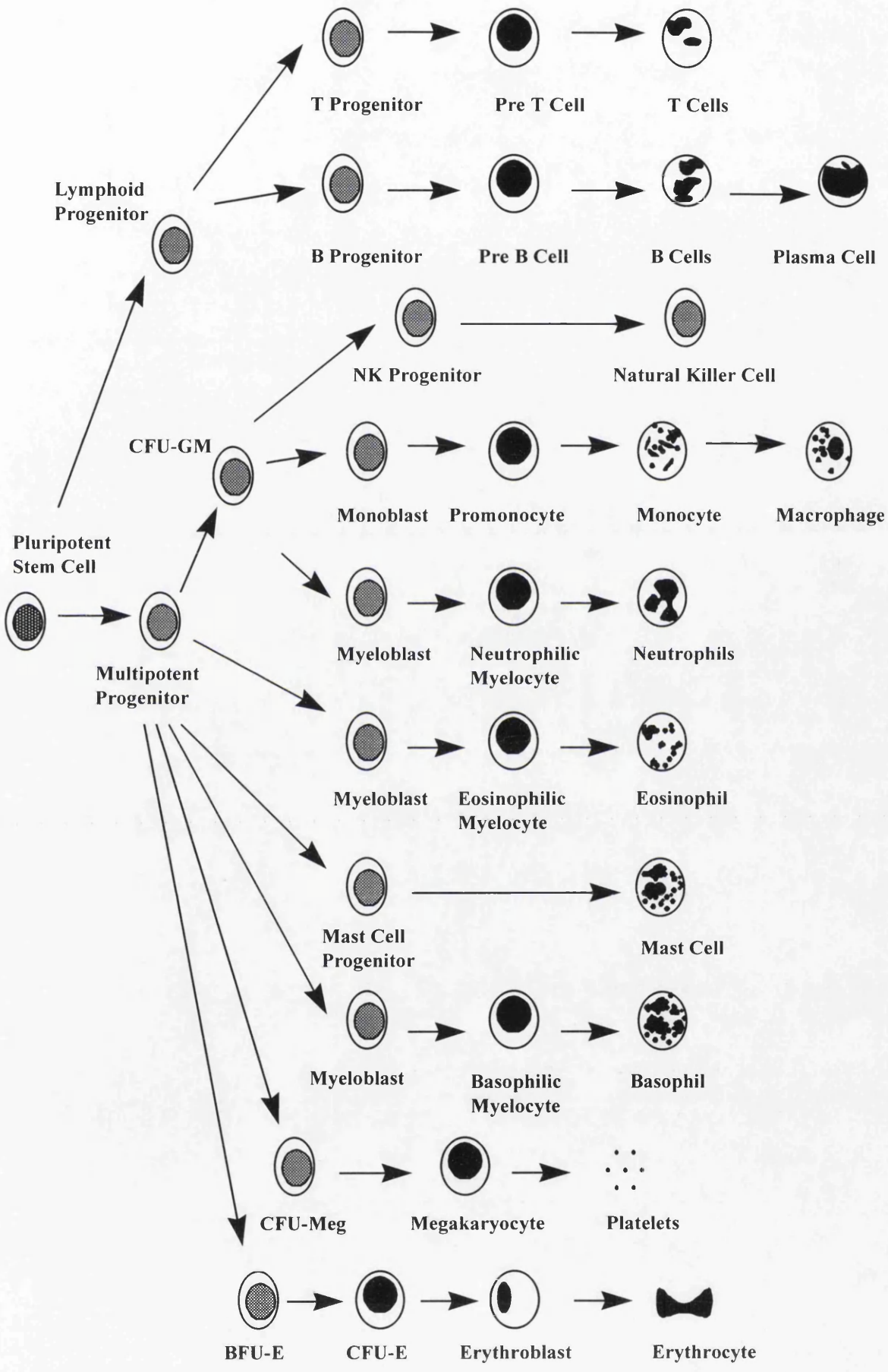
1.2 The Stem Cell Compartment

One can consider the stem cell compartment in its simplest form, as "a heterogeneous compartment consisting of cells displaying varying degrees of self-renewal capacity and/or differentiation potential" (Graham and Pragnell 1992a). This is perhaps best illustrated by considering the haemopoietic system as a three-tiered hierarchical system, analagous to a pyramid, which forms an ordered continuum. At the apex of this pyramid are the most primitive cells with a high capacity for self-renewal, the greatest repopulating ability and which are most resistant to proliferation and differentiation stimuli. As one moves further down the hierachy, the cells exhibit a reduced capacity for self-renewal and increased tendency for differentiation. At the base of this pyramid are the mature cells. It is this remarkable ability to undergo self-

Figure 1.1: Overview of the Haemopoietic System

This figure summarises the ability of the haemopoietic system to generate all the necessary different mature blood cell types. Shown is a single pluripotent stem cell, as well as the various different progenitor cells and their lineages. Progenitors are defined by *in vitro* assays, according to the cell type to which they give rise and are known as colony-forming units (CFU) or burst-forming units (BFU). Abbreviations: E=Erythroid, Meg=Megakaryocyte, GM=Granulocyte/Macrophage. For example, CFU-GM represents a progenitor giving rise to granulocytes and macrophages.

Figure 1.1 : Overview of Haemopoiesis



renewal and generate differentiated progeny which defines a stem cell. Stem cells constitute only a tiny proportion of the total haemopoietic system, probably between 0.01 and 0.1 % of the total bone marrow of an adult mouse, and it is believed that the majority of these are held in a quiescent or G_0 state (Hodgson *et al* 1982, Harrison and Lerner 1991). Stem cells are triggered into an actively cycling state in order to replenish progenitor and mature cell compartments, as a result of stress to the system (e.g. following chemotherapy, or during infection). Following replenishment, the stem cells re-enter quiescence.

Significant progress in understanding the nature of haemopoietic stem cells came from a series of experiments in which it was discovered that transplanted murine bone marrow contained progenitor cells that were able to give rise to individual macroscopic colonies of myeloid, erythroid and megakaryocytic cells on the surface of spleens of lethally irradiated recipient mice (Till and McCulloch 1961). This seminal experiment, defined the first *in-vivo* stem cell assay (the CFU-S assay) and produced the first evidence for primitive quiescent stem cells with a capacity for self-renewal and differentiation. Due to the difficulty in distinguishing stem cells on the basis of their morphology, much of what we now know about stem cells has been derived through the development and usage of such *in vivo* and other, *in vitro*, assays.

1.3 The *In Vivo* CFU-S Assay

The aforementioned colony forming unit-spleen (CFU-S) assay (Till and McCulloch 1961) was also important in demonstrating that stem cells are in fact a heterogeneous population of cells. In this assay, transplantation of donor bone marrow cells to a lethally irradiated mouse resulted in the reconstitution of the ablated haemopoietic system and enabled the mouse to survive an otherwise potentially lethal dose of radiation. During reconstitution, colonies formed on the spleen that were donor-derived and which could be detected at various stages of maturity, depending upon when the CFU-S assay was read (Siminovich *et al* 1964). Colonies were first visible on the spleen at day 7 or 8 and other colonies were visible at a later time, around day 12. Subsequent studies, in which individual day 7/8 spleen colonies were transplanted into a similarly lethally irradiated mouse, demonstrated very little secondary CFU-S colony formation and indicated that the early appearing (day 7/8) colonies were derived from cells with a limited self-renewal/differentiation potential.

Cells derived from individual day 12 colonies were however able to generate multiple secondary CFU-S colonies on serial transplantation, indicating that cells within these later-derived colonies had a greater capacity for self-renewal (Wolf and Priestly, 1986). Put more simply, colonies appearing early at day 7/8 were derived from more mature cells, whilst the later appearing day 12 colonies were derived from more primitive cells. Originally it was assumed that the day 12 colonies, since they appeared later, were derived directly from the earlier appearing day 7/8 colonies by differentiation and self-renewal. However studies have shown that 50 % of the day 7/8 colonies (mostly erythroid in morphology) subsequently regressed, following which, colonies were observed at day 12, which had a multi-lineage constitution (Magli *et al* 1982). The precise origin of the day 12 colonies remains in controversy, with three possible explanations. They may have arisen as a result of re-circulation of CFU-S cells that originally seeded in the bone marrow, but which subsequently moved to the spleen (Van Zant 1984), or a second explanation is that some stem cells have migrated out of the day 7/8 colonies and formed *de novo* colonies, that appear later at day 12. The third and simplest explanation is that the day 12 colonies simply take longer to appear. Additional evidence for the heterogeneity of stem cells, at least within the CFU-S compartment, has arisen through studies using cell cycle specific toxic drugs, such as 5-fluorouracil (5-FU). These studies demonstrated that the day 7/8 CFU-S cells were extremely sensitive to the lethal effects of these drugs, whereas the later day 12 CFU-S cells were less sensitive (Hodgson *et al* 1982). This suggests that the day 7/8 cells are largely in cycle, whilst the day 12 cells are in a more quiescent state and accordingly represent a more primitive population of stem cells.

Following the early work on the CFU-S assay, it was believed that the day 12 CFU-S cell could be the 'ultimate' or 'totipotent' haemopoietic stem cell *in vivo*. Since then however, various lines of evidence have emerged suggesting that the day 12 CFU-S cell is not the ultimate haemopoietic stem cell. Perhaps most obviously, is the inability of the day 12 CFU-S cell to give rise to cells of the lymphopoietic lineages. Also, despite the importance of donor CFU-S cells in the reconstitution of short-term haemopoiesis in a lethally irradiated mouse (Jones *et al* 1989), these cells are unable to establish long-term *in vitro* or *in vivo* haemopoiesis (van der Sluijs *et al* 1990). This would suggest the existence of another more primitive or 'pre-CFU-S' cell, the existence of which was previously suggested (Hodgson and Bradley 1979).

This pre-CFU-S stem cell would have the ability to give rise to both lymphoid and myeloid lineages (from which the more mature CFU-S cell would be derived as a myeloid committed stem cell). To date this cell has not been purified to homogeneity, but it has been further characterised. Pre-CFU-S cells have been enriched for and separated from, CFU-S cells on the basis of its ability to retain the dye Rhodamine 123 (Ploemacher and Brons 1988, 1989). These cells have been termed long-term repopulating cells (LTRC), for their ability to maintain long-term haemopoiesis. These cells alone are not sufficient however, to effectively establish haemopoietic repopulation in a lethally irradiated animal, nor are they radioprotective. CFU-S cells alone will initiate reconstitution, but this will only persist for a short time and mice die within about 4 weeks. Full, long-term reconstitution only occurs with transplantation of both more mature CFU-S type stem cells, as well as the primitive pre-CFU-S stem cells (Jones *et al* 1990). Therefore, in simplistic terms one can subdivide the stem cell compartment into two subcompartments, comprised of the more primitive long-term engrafting stem cells and the short-term or transiently engrafting stem cells (reviewed by Graham and Wright 1997).

1.4 *In vitro* Culture of Haemopoietic Stem Cells

1.4.1 *In vitro* stem cell assays

As mentioned in previous sections, much of what is known about the stem cell compartment has been derived from the development of *in vitro* assays. The basis for these assays is the fact that clonogenic bone marrow cells cultured in the presence of appropriate growth factors give rise to colonies in semi-solid culture, without the addition of supporting stromal cells. The precise combination of cytokines and their concentrations vary from assay to assay. The size, morphology and composition of these colonies can then be assessed to give an indication of the level of primitiveness of the cell from which they were derived. In general, only differentiation along myeloid and erythroid lineages is observed.

A number of *in vitro* assays have been reported, that detect a range of cells within the haemopoietic stem cell compartment. The precise role that many of these cells play in normal steady-state haemopoiesis *in vivo* is still uncertain. These assays have however, enabled identification of several primitive stem cell types and

haemopoietic stem cell regulatory factors. Some of these assays are outlined briefly below and were recently reviewed by Graham and Wright (1997).

The high proliferative potential colony-forming cell (HPP-CFC) was originally identified by Bradley and Hodgson (1979), using a clonal *in vitro* assay that detected macroscopic colonies in agar culture. These HPP-CFC differ from more mature haemopoietic cells, by virtue of their relative resistance to 5-fluorouracil, their requirement for a combination of regulatory growth factors and their ability to form large colonies (>0.5 mm) in agar culture. HPP-CFC have been further defined and now form three distinct populations of stem cells, known as HPP-CFC 1, 2 and 3. Studies with 5-fluorouracil have suggested that HPP-CFC 1 cells are the more primitive, whilst HPP-CFC 2 and 3 are more mature in phenotype. It is believed that the HPP-CFC population forms part of the stem cell compartment detected by the *in vivo* CFU-S assay (reviewed by Bertoncello 1992, Graham and Wright 1997).

The CFU-A assay is an *in vitro* assay that enables detection of primitive progenitor cells, that are HPP-CFC-like and which are believed to be equivalent to the population of cells detected by the *in vivo* day 12 CFU-S assay. The CFU-A assay detects a cell with an incidence of approximately 200 per 1 x 10^5 cells in normal bone marrow and which forms colonies >2 mm in diameter (Pragnell *et al* 1988, Lorimore *et al* 1990).

1.4.2 The stromal microenvironment

The most accurate *in vitro* culture model, of *in vivo* haemopoietic stem cell activity, is obtained through the use of stromal or long-term bone marrow cultures (LTBMC). The bone marrow microenvironment is a complex 3-dimensional structure consisting of a meshwork of heterogeneous cell types and extracellular matrix proteins. It provides an environment that allows the maintenance, self-renewal, proliferation and differentiation of haemopoietic stem cells and ultimately their migration to the circulation (Dexter 1982, Deryugina and Muller-Sieberg 1993). Bone marrow stromal cells are a crucial component in this micro-environment and include macrophages, fibroblasts, adipocytes, and endothelial cells (Gordon *et al* 1980, Mori *et al* 1990, Greenberger 1991). Stromal cells can be grown from freshly isolated bone marrow as *in vitro* cultures and will, under the appropriate conditions, form confluent adherent layers that will support the proliferation, differentiation and limited self-

renewal of haemopoietic progenitors in the absence of added haemopoietic growth factors. The first stromal cultures were developed using murine haemopoietic tissue (Dexter *et al* 1977a, 1977b) and were subsequently adapted to human cells (Gartner and Kaplan 1980). Whilst cells with long-term repopulating ability *in vivo* can be maintained for several weeks *in vitro* (Fraser *et al* 1991), both human and murine stromal-dependent LTBMCs have a limited life span. In these LTBMCs, committed progenitor cells rapidly undergo terminal differentiation and within 4-5 weeks these initial committed progenitor cells have disappeared and at this time, other clonogenic progenitors are detected. These cells are derived from primitive cells, termed long-term culture initiating cells (LTC-IC) (Sutherland *et al* 1989). These LTC-IC appear to be present in normal bone marrow at a frequency of approximately 1-5 per 1 x 10^4 cells and were recently shown to be capable of self-renewal and expansion *in vitro* (Petzer *et al* 1996). Despite their limitations, LTBMC have provided a useful *in vitro* culture system for modelling the structure of the stromal microenvironment and its interactions with haemopoietic stem cells.

1.5 Progenitor Cells and Lineages

Haemopoietic stem cells are able to undergo self-renewal, and this has been postulated to be of two types. 1) Symmetric self-renewal, which results in the generation of two identical daughter stem cells and 2) asymmetric self-renewal, that results in an identical daughter stem cell and a daughter cell that is committed to differentiation along a specific lineage(s). As shown previously in figure 1.1, differentiation occurs through various lineages and via a variety of morphologically identifiable intermediate progenitor cells, ultimately giving rise to terminally differentiated mature cells. These haemopoietic progenitor cells (HPCs) have only a limited capacity for self-renewal and are defined through the ability to give lineage specific progeny in *in vitro* assays. These assays, as those used for HSCs, are based on the ability of progenitor cells to form colonies upon culture in semi-solid agar or upon methylcellulose, in the presence of the appropriate haemopoietic growth factors. These "colony assays" led first to the growth and clonal differentiation of macrophages and granulocytes from their progenitor cells (Paran *et al* 1970, Pike and Robinson 1970) and subsequently to the *in vitro* generation of the majority of other blood cell lineages (Stephenson *et al* 1971, Metcalf *et al* 1975). The HPCs are defined according to the

precursor cell type to which they give rise and are known as colony-forming units (CFU) or burst-forming units (BFU). So for example, cells from early and late progenitors giving rise to colonies containing erythroid cells are known as BFU-E or CFU-E, respectively, whilst those progenitors giving rise to granulocytes or macrophages are called CFU-GM.

These assays have enabled the identification, characterisation and cloning of a multitude of regulatory factors, that have been implicated in the process of stem cell function. The function of some of these regulatory factors and their overall importance to the regulation of haemopoiesis will be discussed in greater detail in chapter 2.

1.6 Inhibition of Haemopoiesis

The existence of a multitude of positively acting or stimulatory haemopoietic growth factors and cytokines, and their potential importance in regulating haemopoiesis, is well documented. What is less certain however, is the role inhibitory molecules play in haemopoiesis. One can argue that the action of positive regulators alone, is sufficient to control the proliferation of haemopoietic stem cells. There are however several problems with this argument. On a superficial level, one can question if this arrangement would provide a sufficiently rapid and tightly controlled regulation. Under normal steady state conditions, it is estimated that only 10 % or fewer of the haemopoietic stem cells are in active cell cycle, with the remainder in a quiescent or G_0 phase. One can envisage that stress to the system, such as that of inflammation, could result in inducible haemopoiesis (see section 1.7) and activation of previously quiescent stem cells. Under circumstances of extreme stress, such as bone marrow regeneration following chemotherapy or radiotherapy, even the most primitive stem cells are actively cycling (Harrison and Lerner 1991). Following activation, the majority of the cells within the haemopoietic stem cell compartment then return to their normal quiescent state. The existence of a variety of inhibitory molecules, that actively inhibit the cell cycle have been proposed to be responsible for the return to, or even maintenance of, stem cell quiescence. Whilst it is possible that the action of stimulatory regulators alone may be sufficient to control haemopoietic stem cell proliferation, the existence of both positive and negatively acting regulators would allow for a far more flexible and responsive system of control. In such a model, positive and negative regulators could both operate, and the relative balance between

their opposing actions would ultimately control the state of haempoietic stem cell proliferation.

Further support for the existence of negative regulators comes from the identification of molecules that have the ability to inhibit stem cell proliferation (reviewed in Axelrad 1990, Graham and Pragnell 1990, Graham 1997). Both novel inhibitory molecules, such as the Tetrapeptide (AcSDKP), and Haemoregulatory peptide (pEEDCK) (both reviewed in Graham and Pragnell 1990, Guigon and Bonnet 1995, Graham 1997) have been identified, as well as previously described molecules such as TGF-β1, MIP-1α and TNF-α. Interestingly some of these 'cytokines' appear to be able to function bi-directionally, in that they can both stimulate as well as inhibit, the growth of some cell types (Keller *et al* 1994). This will be discussed further in sections 2.14.6 and 3.6.5, with regard to the known functions of TGF-β1 and MIP-1α respectively.

1.7 The Stromal Microenvironment and the Production of Soluble Mediators

As mentioned previously, the stromal microenvironment is crucial to the regulatory processes involved in haemopoiesis in the bone marrow. Much of what is known about the potential role of stromal regulation of haemopoiesis has been determined through the investigation of LTBMC and other *in vitro* culture systems designed to mimic the stromal microenvironment. A multitude of studies has been reported, particularly with regard to further understanding the relationship between the stromal cell network and the various cytokines and other soluble mediators implicated in regulating haemopoiesis. At this point, it is worth mentioning inducible haemopoiesis (as opposed to constitutive haemopoiesis), which can be defined as haemopoiesis induced as a result of an increased need for mature blood cells (e.g. during infection and inflammatory responses). Whilst these two forms of haemopoiesis may operate under different circumstances and via different mechanistic routes, a complex network of interacting cytokines and growth factors has been implicated in both. Regardless of which model(s) of haemopoiesis one subscribes to (stochastic or instructive; see sections 2.15.1 and 2.15.2), this network of cytokines is strongly implicated in a multitude of roles, such as the control of mobilisation, self-renewal, survival, proliferation, commitment, differentiation and migration of cells

within the haemopoietic system. Further discussion of the potential role of this network in the control of haemopoiesis is detailed in chapter 2.

1.7.1 Bone marrow and stromal production of cytokines and growth factors

Steady state or constitutive haemopoiesis is regulated in the bone marrow by direct cell-cell contact between bone marrow stroma and haemopoietic stem and progenitor cells, and by locally produced soluble mediators (see figure 1.2).

Direct cell-cell contact may not be a pre-requisite in LTBMC, which mimic the stromal microenvironment, since stromal cells separated from haemopoietic cells by a thin porous mesh can still support haemopoiesis (Verfaillie 1992). This suggests that these stromal cells can produce soluble mediators that enable haemopoiesis to be maintained (Verfaillie 1993, Verfaillie *et al* 1994). Many studies have examined production of such soluble factors both *in vivo* and *in vitro*, as discussed below.

Attempts to detect mRNA expression and protein production has been complicated by the difficulty in detection of these factors and to date the results are still very confused and sometimes even contradictory. Constitutive expression of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) by a variety of cell types, including fibroblasts and bone marrow stromal cells in long-term culture, have been widely reported (Piersma *et al* 1984, Sieff *et al* 1988, Schaafsma *et al* 1989, Kittler *et al* 1992). Detection of constitutive expression of granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and interleukin (IL)-6 mRNA has also been documented (Kittler *et al* 1992, Guba *et al* 1992). The role of IL-3 in the regulation of constitutive haemopoiesis is however, still very confused. Whilst the ability of IL-3 to function as a multilineage colony-stimulating factor during the earlier stages of haemopoietic stem cell differentiation *in vitro* are well documented (Sonoda *et al* 1988, Koike *et al* 1987) attempts to detect constitutive IL-3 expression, without the addition of inducing stimuli, have been largely unsucessful (Guba *et al* 1992, Cluitmans *et al* 1995). Expression of IL-3 has however, been detected in irradiated stromal cells using the sensitive technique of polymerase chain reaction (PCR) amplification and addition of IL-3 specific antibodies resulted in the abrogation of the ability of these stromal cell lines to support the growth of factor dependent cell lines (Kittler *et al* 1992). These results suggest that whilst the presence of growth factors in LTBMC stromal cells and

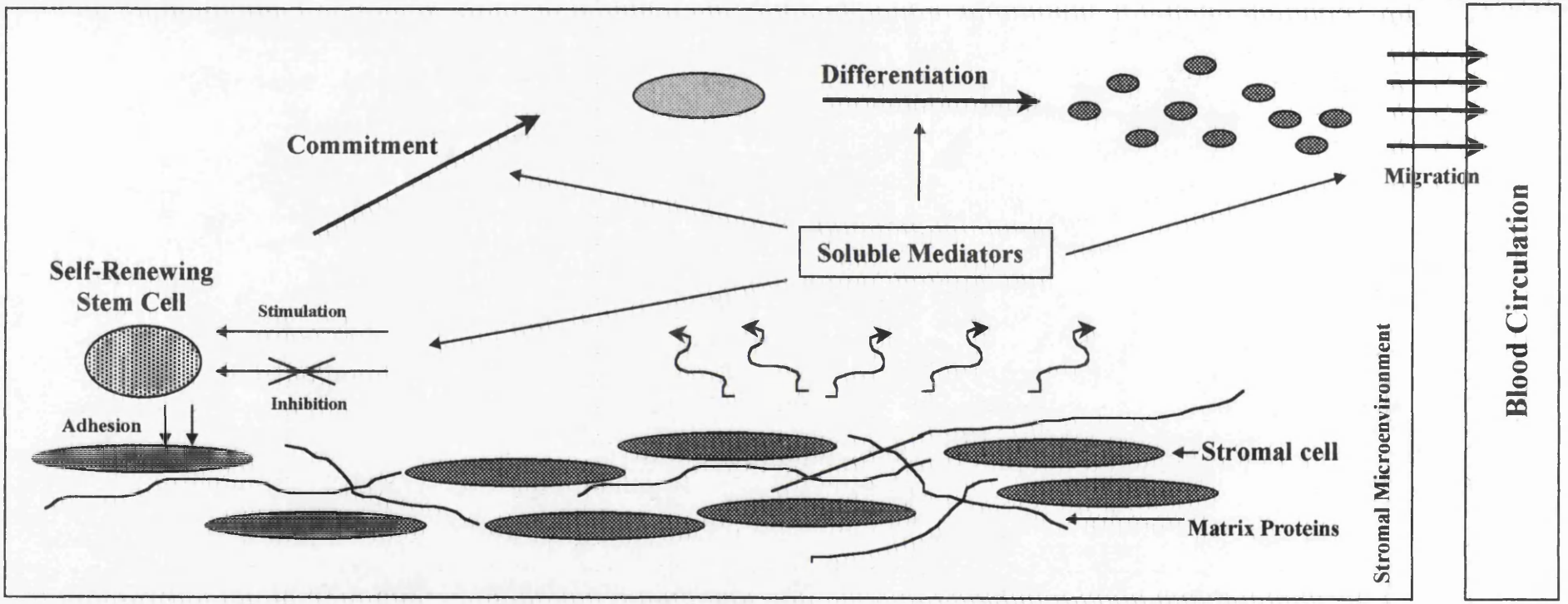


Figure 1.2 : Haemopoiesis and the Stromal Microenvironment

The above figure shows a simple diagrammatic representation of the haemopoietic microenvironment. It summarises some of the processes that occur within the microenvironment, such as those of adhesion, stimulation, inhibition, self-renewal, commitment, differentiation and migration. The production of a variety of soluble mediators is believed to be an important part of the regulation of these processes and their production by stromal cells is also indicated in the above figure.

bone marrow may be undetectable by techniques such as Northern blotting and PCR, their actual presence (albeit at extremely low levels) can not be excluded.

Whilst many reports have documented the constitutive expression of GM-CSF and G-CSF by a variety of cell types crucially important to the bone marrow microenvironment, Cluitmans and his collegues were unable to detect their constitutive expression in normal human bone marrow (Cluitmans *et al* 1995). This study suggested that whilst constitutive expression of the cytokines M-CSF, SCF, IL-1β, IL-4, IL-6, IL-7, tumour necrosis factor-beta (TNF-β), transforming growth factor-beta (TGF-β) and macrophage inflammatory protein-1 alpha (MIP-1α) was detectable in human bone marrow, expression of G-CSF and GM-CSF was not. This raises the question of whether or not GM-CSF and G-CSF play a role in uninduced/normal steady-state haemopoiesis. However, it should be noted that although Cluitmans *et al* were unable to detect constitutive GM-CSF expression in normal bone marrow, this does not preclude their expression at subliminal levels. Indeed, evidence from studies on mice deficient for GM-CSF, suggest that GM-CSF is critical for the homeostasis of normal lung physiology and resistance to local infection (Stanley *et al* 1994) and indicates that at least some subliminal GM-CSF expression must occur *in vivo*. Likewise, studies on mice deficient for G-CSF indicate at least some role for G-CSF in the *in vivo* production of granulocytes (Lieschke *et al* 1994).

1.7.2 Localisation of growth factors

Many soluble and insoluble mediators are produced by the stromal microenvironment, though in many cases, detection of these has proved extremely difficult. This suggests that if some of these factors are indeed produced within the bone marrow, they may be at extremely low levels (perhaps even below levels detectable with current techniques). One possible explanation for this may lie with the apparent existence of growth factor localisation. It is now evident that some cytokines and growth factors (such as SCF, M-CSF and IL-1) exist not just as soluble forms, but also as biologically active membrane or extra-cellular membrane (ECM) bound forms (Kurt-Jones *et al* 1985, Stein *et al* 1990). Other growth factors, e.g. GM-CSF, IL-3 and leukemia inhibitory factor (LIF) are also able to bind to components of the ECM (Gordon *et al* 1987, Roberts *et al* 1988). The ability to bind components of the ECM, as found within the stromal microenvironment, would enable these growth factors to

be held in an active, but highly localised fashion. There is some intriguing evidence that certain components of the ECM, such as heparan sulphate and other glycosaminoglycan (GAG) derivatives, can function directly on their own to regulate growth factor expression (Wrenshall *et al* 1991, Yang and Yang 1995). With the possible exception of fibroblast growth factor (Spivak-Kroizman *et al* 1994), these proteoglycan interactions are clearly not essential for growth factor function as exogenous soluble growth factors will function in the absence of such interactions. This does not however exclude their involvement in a localised niche within the stem cell microenvironment, perhaps in the sequestration or presentation of growth factors.

1.8 Haemopoietic Regulators and the Cytokine Network: Redundancy or Eloquent Control ?

There are a vast number of cytokines and growth factors and some of these, are summarised in table 1.1. Whilst not all play a direct role in regulating haemopoiesis, a large number of them clearly do. Given the known complexity and precision of haemopoiesis, it is not really a surprise that a large number of regulatory molecules have been identified to date and indeed further regulators continue to be identified. What is perhaps surprising, is the seemingly overlapping actions or redundancy of many regulators.

There does at first sight appear to be a great deal of evidence supporting the notion of redundancy (reviewed in Metcalf 1993). It is clear for instance, that more than one factor can stimulate the formation of the same type of colony, e.g. G-CSF, GM-CSF, IL-3, and IL-6 can all function to stimulate formation of granulocytes (Metcalf 1993). A similar number of factors can also stimulate the formation of eosinophil and mast cell colonies. Such an arrangement is seemingly wasteful and redundant, as well as offering a greatly increased risk of dysregulation. A second argument is that certain regulators, particularly LIF, IL-6 and IL-11 (Metcalf 1991, Kishimoto *et al* 1995), appear to have an unusually wide range of target cells. In many cases these cytokines also exhibit (at least partial) overlaps in their actions.

It is now evident that the receptors for these cytokines and growth factors can be subdivided into 3 major categories (reviewed in Park *et al* 1990a, Ullrich and Schlessinger 1990, Cosman *et al* 1990, Bazan 1990, Hara *et al* 1996, Bagley *et al* 1997), as follows: 1) Receptors belonging to the immunoglobulin superfamily, e.g.

Table 1.1: Summary of Cytokines and Growth Factors

Interleukins and Colony-Stimulating Factors

IL-1α	IL-10	G-CSF
IL-1β	IL-11	GM-CSF
IL-2	IL-12	M-CSF
IL-3	IL-13	SCF
IL-4	IL-14	LIF
IL-5	IL-15	Epo
IL-6	IL-16	TPO
IL-7	IL-17	TNF-α
IL-8	IL-18	TNF-β
IL-9		Ftl3L
		CNTF
		OSM

TGF-β superfamily	Interferons	Other Growth Factors
TGF-β1	IFN-α	EGF
TGF-β2	IFN-β	TGF-α
TGF-β3	IFN-γ	acidic FGF
Inhibin		basic FGF
Activin		PDGFα
Bone Morphogenetic Proteins (BMPs)		PDGFβ
		IGF-I
		IGF-II
		NGF
		HGF

Chemokines

See Table 3.1 for a full list of Chemotactic cytokines (Chemokines)

Abbreviations used:

IL-, interleukin; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; Epo, erythropoietin; FGF, fibroblast growth factor; Ftl3L, Ftl3 ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IGF, insulinlike growth factor; IFN, interferon; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; NGF, nerve growth factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; SCF, stem cell factor; TGF, transforming growth factor; TNF, tumour necrosis factor; TPO, thrombopoietin.

IL-1R; 2) the tyrosine kinase receptors, which include receptors for M-CSF and SCF; and 3) receptors belonging to the "cytokine receptor superfamily", that are subdivided further on the basis of homology and which includes the haemopoietin receptors. Given their homology, it is almost certain that evolution of some of these receptors, has arisen through duplication of a common ancestral gene. This is most evident in the structure of the haemopoietin receptors. Many are heterodimers, with an α-chain specific for the cytokine cognate to that receptor and a β-chain that is shared among members of the same subfamily. For instance GM-CSF, IL-3 and IL-5 share a common β-chain (reviewed in Miyajima *et al* 1993). This β-chain is proposed to transduce the signal from the receptor complex to the intracellular compartment and may explain why GM-CSF, IL-3 and IL-5 all show the overlapping ability to stimulate eosinophil proliferation and functions. A similar situation exists for IL-6, IL-11, oncostatin M (OSM) and LIF, which share a common subunit chain (the gp130 subunit) and this is a probable explanation for the appearance of many of the overlapping pleiotropic functions of these cytokines (reviewed in Kishimoto *et al* 1995).

A multitude of studies has now been reported in which individual (and even combinations of) specific growth factors or cytokines have been deleted in mice. The various phenotypes of these mice, suggest that whilst some facets of their function may overlap, others are clearly unique. As indicated above, M-CSF, G-CSF, IL-3, SCF and IL-6 can all stimulate the formation of granulocytes. However, the gene for each of these has been separately deleted in mice and each shows a very distinct phenotype. Such deletion analysis has suggested functions for these molecules in both constitutive and inducible haemopoiesis, i.e. distinct and non-redundant roles. This suggests, that to say that some factors are redundant is over simplistic. Indeed, there is another alternative to redundancy as an explanation for the plethora of regulatory immuno-haemopoietic cytokines. A complex system such as haemopoiesis must be inherently flexible, in order to be able to respond rapidly to a multitude of sudden stresses to the system. A network of interacting cytokines allows such flexibility and enables rapid, yet controlled responses. From an evolutionary point of view this makes more sense too, as a lack of such flexibility could prove lethal. A network with overlapping functions also provides a fail-safe mechanism, by which one cytokine can substitute for another should that one not function properly.

There are clearly many other logical reasons why a such a complex interactive network makes sense. It can provide an in built amplification mechanism, whereby one cytokine has the ability to induce the expression of other cytokines and even autoregulate its expression (Lotem and Sachs 1991).

An additional level of control exists, with the potential to couple cytokine expression to that of cytokine receptor expression (Walker *et al* 1985). For instance IL-6 induces expression of receptors for M-CSF and IL-3 (Lotem *et al* 1989) and TGF-β downregulates expression of receptors for GM-CSF, G-CSF and IL-3 (Jacobsen *et al* 1991a).

Clearly, inducing or stimulating factors are only part of the cytokine network and the network is in fact likely to be a balance between these positive signals and those exerted by molecules with inhibitory functions, such as TGF-β, TNF-α and MIP-1α. The role of inhibitory regulators in haemopoiesis was briefly discussed in section 1.6, whilst a discussion of the inhibitory roles of TGF-β and MIP-1α can be found in sections 2.14.6 and 3.6.5 respectively. The following sections of chapter 2 summarise the roles and importance of some of the growth factors and cytokines (previously indicated in table 1.1), which together form an interacting network of regulatory molecules implicated in the survival, growth, differentiation and function of cells within the immuno-haemopoietic system.

CHAPTER 2: Growth Factors and Cytokines

This chapter will focus on some of the key regulators of the immuno-haemopoietic system and discuss some of their known functions. This can not possibly be an exhaustive review, due to the overwhelming literature available. Instead, it is intended to familiarise the reader with some of the most important points concerning the role of specific growth factors and cytokines, in the network of cytokines that is implicated in the regulation of the immuno-haemopoietic system.

2.1 Interleukin-1 (IL-1)

IL-1 is a cytokine responsible for mediating a variety of processes in host defence, inflammation and haemopoiesis. IL-1 is most closely related to tumour necrosis factor (TNF), but is a member of a small cytokine subfamily that includes IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1 was originally described as a product of macrophages, but is now known to be produced by a variety of cell types, including fibroblasts, keratinocytes, T and B lymphocytes, astrocytes and microglial cells. IL-1α and IL-1β are two related, but distinct molecules, that are synthesised as 31 kD precursors (proIL-1α and proIL-1β). Unlike proIL-1α, pro-IL-1β requires cleavage for optimal secretion and activity and this occurs through the action of IL-1β converting enzyme (ICE) (Thornberry *et al* 1992, Wilson *et al* 1994). Whilst both IL-1α and IL-1β exist as extracellular proteins, IL-1α can also exist as a membrane bound form, that is also biologically active (Matsushima *et al* 1986, Kurt-Jones *et al* 1985). In most tissues, IL-1β mRNA predominates over IL-1α mRNA and in fact IL-1α is rarely found in the circulation. The many inflammatory functions of IL-1 will not be discussed further and the reader is referred to reviews on this subject, such as that by Dinarello (1994).

IL-1 affects various levels of haemopoiesis and its direct haemopoietic effects were first identified as hemopoietin-1, an activity that modulated the response of very primitive progenitor cells to colony-stimulating factors (Stanley *et al* 1986, Mochizuki *et al* 1987). IL-1 induces the production of GM-CSF, G-CSF, M-CSF and IL-3 and other cytokines, particularly from bone marrow stromal cells (Zucali *et al* 1986). IL-1 has been shown to act synergistically with IL-3, IL-6, G-CSF and GM-CSF, to induce

single and multi-lineage colony formation (Moore *et al* 1987, Bradley *et al* 1989). *In vivo*, IL-1 has been shown to protect stem cells and promote granulopoiesis following cytotoxic drug treatment or irradiation (Fibbe *et al* 1989, Oppenheim *et al* 1989). IL-1β also appears to stimulate platelet production *in vivo*, as shown in a murine model (Williams and Morrissey 1989). *In vivo* experiments have also suggested that IL-1 can function as an inhibitor of progenitor cell proliferation. IL-1α reversibly inhibits late-stage erythropoiesis (Johnson *et al* 1989) and prolonged administration of IL-1β has been shown to inhibit the formation of granulocyte-macrophage (GM) colonies, albeit indirectly through induction of TNF-α, a known inhibitor (Gasparetto *et al* 1989).

2.2 Interleukin-3 (IL-3)

During the search for colony-stimulating activities, several activities were found in spleen conditioned medium. These activities were designated by various names, including erythroid burst (colony)-promoting activity (BPA), mast cell growth factor (MCGF), Thy-1-inducing activity, hemopoietin-2, 20αhydroxysteroid dehydrogenase (20αSDH) inducing activity, and interleukin-3. These activities were suggested in fact, to be the result of a single colony-stimulating factor and were subsequently re-named multi-CSF, which is now more commonly known as IL-3. Unusually, IL-3 appears to be potentially involved in regulating the production of all the major myeloid blood cell types, including macrophages, megakaryocytes, mast cells, eosinophils, neutrophils and erythrocytes.

The major source of IL-3 appears to be antigen or mitogenically activated T lymphocytes. However, in addition to activated T cells, NK cells, (Cuturi *et al* 1989), mast cell lines (Wodner-Filipowicz *et al* 1989), megakaryocytic cell lines (Avaraham *et al* 1992) and thymic epithelial cells (Dalloul *et al* 1991) have been shown to express IL-3. Reports of IL-3 expression in cells within the bone marrow microenvironment, such as fibroblasts and endothelial cells have largely been negative (Cluitmans *et al* 1995) although one study, using a sensitive PCR-based detection method, did report the detection of IL-3 expression in irradiated bone marrow (Kittler *et al* 1992). The persistent failure to detect IL-3 in either bone marrow or normal serum has led to the suggestion, that IL-3 does not in fact play a role in constitutive haemopoiesis. Instead,

some researchers have suggested that it functions as a mediator of inflammation, due to its immune cell-derived sources.

Whilst the precise role of IL-3 *in vivo* remains controversial, the *in vitro* ability of IL-3 to function as a multilineage colony-stimulating factor is well documented. *In vitro,* recombinant IL-3 induces the formation of colonies in cultures of bone marrow cells in soft agar. It promotes the formation of mixed erythroid/myeloid, macrophage, neutrophil, eosinophil and basophil colonies (Hapel *et al* 1985, Rennick *et al* 1985). IL-3 has also been reported to support the growth of erythroid and megakaryocyte lineages, but the formation of mature cells does not occur in the absence of erythropoietin or thrombopoietin. A number of reports have provided evidence that IL-3, along with GM-CSF, is more specific for early stages of haemopoietic development. For instance, IL-3 is required for the proliferation and differentiation of multipotential haemopoietic progenitors in culture, though IL-3 does not appear to promote the entry of these quiescent progenitor cells into active cell cycle (Suda *et al* 1985). It has also been reported that multipotent progenitors become less sensitive to IL-3 as they mature into multilineage colonies (Koike *et al* 1986). Taken together these, and other studies, suggest that *in vitro,* IL-3 acts primarily on multipotent progenitors in the earlier stages of haemopoietic development.

IL-3 can clearly also function through synergy with other growth factors. For example, IL-3 synergises with M-CSF, to enhance M-CSF receptor levels and generate 'giant' macrophage colonies from HPP-CFCs, a progenitor cell with high proliferative potential (see section 1.4.2) (McNiece *et al* 1984, Bartelmez *et al* 1985). IL-3 has also been reported to act in synergy with IL-6 to enhance IL-3 dependent proliferation of multipotential haemopoietic progenitors (Ikebuchi *et al* 1987) and with IL-4 to enhance mast cell growth (Smith *et al* 1986). Interestingly, further insight into the role of IL-3 synergy with other growth factors has come from the study of particular mouse strains (mostly A/J, AKR and NZB), which appear to be less responsive or 'hyporesponsive' to IL-3 (Kincade *et al* 1979, Morris *et al* 1990). Progenitor cells from these mice strains are defective in their ability to form colonies or proliferate in response to IL-3, and yet they are still able to exhibit a synergistic response to IL-3 in combination with M-CSF, indicating that responses to IL-3 are not totally absent in these strains. (Breen *et al* 1990, Morris *et al* 1990). The genetic basis for this hyporesponsiveness has recently been identified, as being due to a defect in the

gene encoding the α chain for the IL-3 receptor (IL-3Rα). Unusually however, this defect is not absolute and some functional receptors do appear to be expressed by haemopoietic bone marrow derived mast cells. (Leslie *et al* 1996). Further study of such hyporesponsive strains may well provide further insight into mechanisms by which IL-3 is able to synergise with other growth factors.

As discussed above, IL-3 is almost undetectable under most circumstances and many *in vivo* studies have proved confusing and difficult to interpret. Some have however, proved useful and suggest that there is at least some potential role for IL-3 *in vivo*. For instance, continuous infusion of IL-3 into mice was shown to result in increased numbers of progenitor and myeloid committed precursor cells in the spleen, but not in the bone marrow (Kindler *et al* 1986). Similar results were obtained by intraperitoneal injection of IL-3, which resulted in large increases in macrophage, eosinophil and neutrophil cell numbers, as well as enhanced peritoneal phagocytic activity (Metcalf *et al* 1986). It is possible however, that these changes could be attributable to chemotaxis and mobilisation of cells from the circulation in response to an IL-3 induced mediator, suggesting a role for IL-3 in inflammatory processes. Some evidence for a role for IL-3 to function *in vivo* in constitutive haemopoiesis came from studies using mice that had been pre-treated with purified lactoferrin, (Broxmeyer *et al* 1987a) which has been shown to suppress release of some haemopoietic growth factors *in vitro* and to inhibit myelopoiesis *in vivo*. In this way, the authors were able to suppress the background of normal haemopoiesis and demonstrate that a small infusion of IL-3 resulted in an change in cell cycle status and increased the number of progenitor cells in both the bone marrow and the spleen. In another study, the authors were also able to demonstrate that IL-3 could synergise with GM-CSF and M-CSF *in vivo*, to increase the number of progenitor cells and the absolute number of progenitor cells (Broxmeyer *et al* 1987b).

Whilst the effects of IL-3 appear to predominantly affect the early stages of haemopoiesis, effects on mature, fully differentiated cells are also evident. For instance, IL-3 can potentiate mature eosinophil functions, such as degranulation (Fujisawa *et al* 1990) and stimulate histamine release from basophils (Kurimoto *et al* 1989). IL-3 has also been reported to affect the functions of mature monocytic phagocytes, such as cytotoxicity (Cannistra *et al* 1988), as well as stimulating macrophage growth outside the bone marrow (Chen *et al* 1988).

The *in vivo* role of IL-3 may however not be essential, as mice deficient in the βc or β_{IL-3} components of the IL-3 receptor exhibit apparently normal haemopoiesis (Nicola *et al* 1996), as do mice completely lacking IL-3 functions (Nishinakamura *et al* 1996a). The role of IL-3 *in vivo* therefore remains controversial.

2.3 Interleukin-4 (IL-4)

IL-4 was initially characterised as B cell growth factor (BCGF), subsequently renamed B-cell stimulatory factor and is now known as IL-4. The main function of IL-4 appears to be in regulating the switch in immunoglobulin (Ig) synthesis to IgE (and IgG1 in mice), although it clearly has many other pleiotropic functions (reviewed in Paul 1991). Besides regulation of Ig class-switching, IL-4 can also function as a haemopoietic regulator. It has been shown to enhance the IL-3 mediated growth of mast cells (Mosmann *et al* 1986). IL-4 acts as a co-stimulator with other haemopoietic growth factors, such as G-CSF, erythropoietin (Epo) and IL-6, inducing the proliferation of CFU-GM, BFU-E and CFU-GEMM (Rennick *et al* 1987, Peschel *et al* 1987, Sonoda *et al* 1990).

Several inhibitory actions have been described for IL-4, such as macrophage colony formation (Jansen *et al* 1989) and IL-4 has also been shown to induce expression of a reversible inhibitory activity from bone marrow stromal cells (Peschel *et al* 1989). Taken together, these studies suggest that IL-4 has a potential role in the regulation of various stages of haemopoiesis.

2.4 Interleukin-5 (IL-5)

IL-5 is an unusually specific growth factor, directed to cells of the eosinophilic and basophilic lineage. In fact, IL-5 appears to be the most important, if not only cytokine involved in eosinophilia (elevated level of eosinophils). Eosinophils are cytotoxic cells capable of killing larger organisms such as helminths, parasitic protozoa, as well as tumour cells and normally constitute a very small population within the immuno-haemopoietic system. In addition to its many eosinophil specific functions (reviewed by Sanderson 1992), IL-5 also has actions on mature basophils (reviewed by Denburg 1992).

IL-5 was first identified as eosinophil differentiation factor and subsequently re-named IL-5. In comparison to GM-CSF and IL-3, IL-5 is a poor stimulator of

colony formation in semi-solid assays, whereas IL-5 is much better at stimulating precursor formation in liquid cultures than either GM-CSF or IL-3. In fact whilst GM-CSF and IL-3 can both generate precursors for several different lineages, including eosinophils (see sections 2.2 and 2.11), only IL-5 is specific for eosinophil progenitor formation (Lopez *et al* 1986, Clutterbuck *et al* 1988). It is speculated that IL-5 is only a late acting factor, as suggested by several studies (Sanderson *et al* 1985). GM-CSF and IL-3 can synergise with IL-5 to induce eosinophils (Lu *et al* 1990, Clutterbuck *et al* 1990, Ema *et al* 1990b).

Whilst *in vitro* studies have been unable to demonstrate conclusively, at what stage of progenitor cell IL-5 acts upon, other studies have clearly demonstrated the fundamental importance of IL-5 *in vivo*. Administration of anti-IL-5 antibody to parasite or helminth infected mice prevented the associated eosinophilia completely (Coffman *et al* 1989), whilst another study showed that retrovirally mediated overexpression of IL-5 resulted in long-lasting eosinophilia (Vaux *et al* 1990). Several studies with IL-5 transgenic mice have also determined the *in vivo* importance of IL-5 to eosinophil production (Dent *et al* 1990, Tominaga *et al* 1991). The study of IL-5 transgenic mice has also supported the notion that IL-5 may act upon B lymphocyte development in the mouse system. Mouse IL-5 had previously been reported to function as a promoter of B cell growth and differentiation (Matsumoto *et al* 1987). Transgenic mice with IL-5 coupled to the metallothionein promoter (mMT-I), showed eosinophilia, but in addition had elevated levels of serum IgM and IgA and an expansion of Ly-1^+ and Ly-1dull $B220^+$ cells in the spleen (Tominaga *et al* 1991). Ly-1^+ cells are a minor population of B cell lineage cells, showing a restricted Ig repertoire. In contrast to mouse IL-5, human IL-5 has not been reported to act on B-cells (Clutterbuck *et al* 1987).

Deletion of both the IL-3 ligand and βc common receptor subunit in mice, removes all ability to respond to GM-CSF, IL-3 and IL-5. Analysis of such mice revealed that these mice exhibited a reduced number of eosinophils and eosinophilic responses to parasitic infections and this was attributable solely to the absence of IL-5 (Nishinakamura *et al* 1996a).

Many questions concerning the role of IL-5 in haemopoiesis remain to be answered and in particular, whether IL-5 is sufficient on its own to stimulate

eosinophil progenitor formation or if it must act in synergy with the earlier acting growth factors, GM-CSF and IL-3.

2.5 Interleukin-6 (IL-6)

IL-6, like many other cytokines, exhibits many pleiotropic functions. It was originally identified as a B-cell differentiation factor (BSF-2), that induced maturation of B cells into antibody producing cells (Hirano *et al* 1985, 1986). As well as effects on B cells, IL-6 also acts on T cells, hepatocytes and neuronal cells (reviewed in Kishimoto 1989, Kishimoto *et al* 1992). IL-6's functions as a potent growth factor for human myeloma cells and as a potential autocrine regulator of myeloma, have also been particularly well studied (reviewed in Klein *et al* 1995).

In addition to its roles in human myeloma, IL-6 has also been well characterised as a regulator of haemopoietic progenitor cells. IL-6 synergises with IL-3, to induce the expansion of haemopoietic progenitor cells (Ikebuchi *et al* 1987, Leary *et al* 1988). IL-6 has also been shown to interact with IL-4 or G-CSF to increase GM colony formation (Rennick *et al* 1989), suggesting that IL-6 is able to interact with a variety of factors to regulate the growth of progenitor cells at different stages of lineage commitment and maturation. A role for IL-6 has also been proposed, in the maturation and production of megakaryocytes (Ishibashi *et al* 1989, Suematsu *et al* 1989) and *in vivo* administration of recombinant IL-6 in primates has been demonstrated to result in an increase in the number of platelets (Asano *et al* 1990).

Interestingly, analysis of IL-6 deficient mice has shown that they exhibit defective leukocyte recruitment, and this appears to be associated with a reduced level of chemokine expression (Romano *et al* 1997). This implies that IL-6 may also be involved in amplifying leukocyte accumulation at sites of inflammation *in vivo*.

2.6 Interleukin-7 (IL-7)

IL-7 is a cytokine that is more restricted in its effects, than most other haemopoietic growth factors. IL-7 was originally described as a stroma-derived pre-B cell growth factor (Hunt *et al* 1987). Subsequently to this, IL-7 has been demonstrated to exert it's proliferative effects on B cell progenitors (Namen *et al* 1988, Suda *et al* 1989, Tushinski *et al* 1991), as well as stimulating T cell progenitors (Conlon et al 1989) and mature T cells (Morrissey *et al* 1989). For a long time therefore, IL-7 was

regarded as being purely a lymphoid-lineage-restricted cytokine. Only recently has a role for IL-7 in myelopoiesis emerged, with the demonstration that IL-7 directly enhances colony-stimulating factor and SCF-induced *in vitro* myeloid colony formation from murine bone marrow progenitor cells (Jacobsen *et al* 1993, 1994a and 1994b). IL-7 has also been shown to induce cytokine secretion from monocytes (Alderson *et al* 1991) and bone marrow macrophages have also been reported to express high numbers of IL-7 receptors (Park *et al* 1990b), suggesting that IL-7 may exert effects on more mature myeloid cells and its effects may therefore be more pleiotropic than originally thought.

2.7 Interleukin-8 (IL-8)

IL-8 although designated an interleukin, is actually a member of the chemokine superfamily of cytokines and will therefore be discussed later in section 3.2.1.

2.8 Interleukin-11 (IL-11)

IL-11 was originally cloned from a primate stromal cell line (Paul *et al* 1990) and appears relatively pleiotropic in its effects, on a wide range of cell types. Only a brief synopsis is detailed here and the reader is refered to the various reviews on IL-11 (Du and Williams 1994, 1997).

IL-11 acts synergistically with other early and late acting growth factors, such as IL-3, IL-4, SCF, flt3 ligand (ftl3L) and GM-CSF (Musashi *et al* 1991, Leary *et al* 1992, Lemoli *et al* 1993, Jacobsen *et al* 1995a), to stimulate various stages and lineages of haemopoiesis. It acts with IL-3, thrombopoietin (TPO) or SCF to synergistically stimulate both murine and human megakaryocytopoiesis and thrombopoiesis (Broudy *et al* 1995). IL-11 can also stimulate various stages of erythropoiesis either on its own, or in synergy with IL-3, SCF or Epo (Quesniaux *et al* 1992). IL-11 has also been shown to affect the differentiation and maturation of myeloid progenitor cells. A protective role for IL-11 has also been well documented. For instance, IL-11 has been shown to provide protection of small intestinal clonogenic stem cells against 5-fluorouracil treatment and irradiation (Potten 1995, 1996). In combination with growth factors such as SCF, IL-13, IL-4 and G-CSF, IL-11 effects on B cell growth have also been reported (Hirayama *et al* 1992, 1994).

2.9 Macrophage Colony-Stimulating Factor (M-CSF)

Colony-stimulating factor-1 (CSF-1) or M-CSF, was originally purified on the basis of its ability to stimulate the growth and differentiation of macrophages (Stanley and Heard 1977). M-CSF is somewhat more complex in its gene structure and regulation of expression at RNA and protein levels, than most other cytokines. Soluble M-CSF is a homodimeric glycoprotein and human M-CSF is encoded by a single gene that is transcribed to give a primary mRNA transcript, that is in turn subsequently alternatively spliced and results in mRNAs encoding different forms of M-CSF. These precursor forms of M-CSF are then proteolytically processed to give various forms of the soluble growth factor (Ladner *et al* 1987). At least two different forms of precursor protein exist, the larger of these is rapidly processed and does not give rise to cell surface M-CSF (Wong *et al* 1987, Rettenmier and Roussel 1988). The smaller precursor is processed however (Kawasaki *et al* 1985), but does not always undergo efficient cleavage and may instead be stably expressed on the cell surface (Rettenmier *et al* 1987). The membrane expressed precursor is then slowly cleaved to yield the soluble homodimeric growth factor. The cell surface form also appears to be biologically active (Stein *et al* 1990).

M-CSF stimulates the growth, differentiation and proliferation of cells of the monocyte/macrophage lineage (Ralph and Nakoinz 1987). Recombinant M-CSF has been shown to stimulate differentiation of progenitor cells into monoblasts (Becker *et al* 1987) and also to induce maturation into promonocytes and monocytes (Rosenfeld *et al* 1990). The effects of M-CSF on human macrophage colony formation *in vitro* do however appear somewhat limited, as GM-CSF and other growth factors have been shown to be necessary for optimal colony formation (Caracciolo *et al* 1987, Zhou *et al* 1988). A role for M-CSF does appear to exist *in vivo* however, as demonstrated by studies using mice with a homozygous null mutation for the *op* mutation. These (*op/op)* or osteopetrotic mice, suffer congenital osteopetrosis as a result of a deficiency in osteoclasts and macrophages. It is now clear that this deficiency in osteoclast formation, lies with a complete absence of M-CSF (Wiktor-Jedrzejczak *et al* 1990) and furthermore, that the *op* mutation lies within the coding region for the M-CSF gene (Yoshida *et al* 1990). Addition of recombinant M-CSF has been shown to correct the haemopoietic abnormalities (Wiktor-Jedrzejczak *et al* 1991), which are also progressively corrected as the mice age (Begg and Bertoncello 1993). There is also

evidence that M-CSF has roles in reproduction, as female *op/op* mice show impaired fertility (Pollard *et al* 1987, 1991).

In addition to its roles on progenitor cells, M-CSF also exerts a variety of actions on mature cells of the monocyte/macrophage lineage, including phagocytic activity and microbial killing (Karbassi *et al* 1987) and macrophage-mediated tumour cell cytolysis (Wing *et al* 1982). M-CSF also induces expression of many cytokines from monocytes and macrophages, including G-CSF, GM-CSF, IFN-γ, TNF and IL-1 (Moore *et al* 1980, Warren and Ralph 1986, Motoyoshi *et al* 1989).

2.10 Granulocyte Colony-Stimulating Factor (G-CSF)

G-CSF is a growth factor that regulates the production of neutrophilic granulocytes. These are a critical component to the host defence system and it is estimated that as many as 120 billion granulocytes are produced each day, simply to maintain normal numbers and this number may increase ten-fold during infection (Demetri and Griffin 1991). Whilst G-CSF activity was identified by many researchers, it was not until 1983 that G-CSF was purified and characterised (Nicola *et al* 1983) and the cDNA for a human G-CSF subsequently cloned (Souza *et al* 1986). G-CSF is produced, in many cases constitutively, by various cell types. Cells of the monocyte/macrophage lineage are a particularly dominant source of G-CSF, along with cells such as vascular endothelial cells and fibroblasts. G-CSF production can be induced by a variety of stimuli, including lipopolysaccharide (LPS) and many other growth factors (Oster *et al* 1989, Vellenga *et al* 1988). Evidence for the role of G-CSF *in vivo*, has come from analysis of mice carrying a homozygous null mutation for G-CSF. These mice have chronic neutropenia, with only approximately 20 % of normal circulating neutrophils and a reduced number of neutrophilic granulocyte and macrophage precursors in their bone marrow (Lieshcke *et al* 1994). This study, suggests that some role for G-CSF in the production of granulocytes does exist *in vivo*, although it is one which can be at least partially fulfilled by another factor(s), since G-CSF$^{-/-}$ mice are viable and do still have some neutrophils. This role may be minimal and G-CSF might function instead, to increase neutrophil production during periods of stress, such as during infection. In normal haemopoiesis, the effects of G-CSF are limited to cells of the neutrophilic lineage. These effects will be discussed

only briefly here and the reader is referred to the many reviews on G-CSF (Nicola 1990, Demetri and Griffin 1991).

In vitro, G-CSF stimulates proliferation and differentiation of neutrophil colony-forming cells, as well as affecting several functions of mature neutrophils (Metcalf and Nicola 1983). G-CSF, acts on a relatively mature progenitor cell population, that is mostly committed to neutrophilic differentiation (Ema *et al* 1990a). Whilst the effects of G-CSF alone are relatively limited, in combination with other growth factors, G-CSF can stimulate the survival and proliferation of multi-lineage progenitor cells *in vitro* (Metcalf and Nicola 1983, McNiece *et al* 1989). In fact, G-CSF was originaly described as a multi-lineage-stimulating or pluripotent growth factor. However, when accessory cells were depleted from culture this pluripotent activity was shown to disappear (Strife *et al* 1987). G-CSF has been shown to exert synergistic or enhancing actions with IL-3 (Ikebuchi *et al* 1988), IL-4 (Rennick *et al* 1987, Peschel *et al* 1987), IL-1α (Moore and Warren 1987), and IL-6 (Rennick *et al* 1989).

In addition to stimulating proliferation of progenitor cells, G-CSF also enhances the functions of mature neutrophil effector cells. These range from 'priming' of cells for receptor-mediated responses (Yuo *et al* 1989), production of cytokines such as interferon-α (Shirafuji *et al* 1990), and enhancement of cytotoxic functions (Lopez *et al* 1983).

G-CSF is now widely used clinically to increase neutrophil numbers and to enhance neutrophil responses, during or following chemotherapy or radiotherapy, or following bone marrow transplantation, thus providing a protective role against opportunistic infections.

2.11 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

GM-CSF, along with G-CSF, M-CSF and IL-3, is one of a number of colony stimulating factors identified to date. It derives its name from the ability to stimulate formation of macroscopic colonies containing neutrophilic granulocytes (neutrophils), eosinophilic granulocytes (eosinophils), and macrophages, or a mixture of these cell types. GM-CSF stimulates the proliferation and maturation of myeloid progenitor cells, as well as having many actions on mature neutrophils, eosinophils and macrophages.

Many different cell types have been reported to produce GM-CSF. These include both immune cells such as T lymphocytes, macrophages and mast cells, as well as adherent cells within the bone marrow microenvironment such as macrophages, fibroblasts and endothelial cells. B lymphocytes and natural killer (NK) cells have also been reported to synthesise GM-CSF (Pistoia *et al* 1987, 1989). The precise nature of this expression is sometimes contradictory, but in most cases GM-CSF does not appear to be constitutively expressed and is instead induced by a variety of stimuli. LPS, IL-1 and TNF-α are amongst the most prevalent and dramatic inducers of GM-CSF expression (Broudy *et al* 1986, Chan *et al* 1986, Sieff *et al* 1988), particularly within the bone marrow microenvironment. In addition to growth factors, other stimuli such as FCS, phagocytosis (Thorens *et al* 1987), and $Fc_{\varepsilon}RI$ triggerring by IgE plus antigen in mast cells (Wodnar-Filipowicz *et al* 1989) have also been demonstrated to induce GM-CSF expression.

GM-CSF was first purified from mouse lung-conditioned medium and found to stimulate proliferation and differentiation of myelomonocytic progenitors (Burgess *et al* 1977). Following identification of the complementary cDNA and generation of recombinant protein, the actions of GM-CSF on hemopoietic cells have been studied in great detail (reviewed in Gough and Nicola 1990, Gasson 1991, Baldwin 1992). *In vitro,* GM-CSF is now known to be a mutilineage stimulator for CFU-granulocyte (CFU-G), CFU-GM, CFU-macrophage (CFU-M), and CFU-eosinophil (CFU-Eo) (Tomonaga *et al* 1986). GM-CSF may also support the growth of erythroid and megakaryocytic progenitors *in vitro* (Sieff *et al* 1985, Emerson *et al* 1989).

The effects of GM-CSF on differentiation and maturation are however, less clear. Several studies have suggested that GM-CSF can function to induce differentiation, for instance towards monocytic and eosinophilic lineages in the human myeloid leukemia cell line HL60 (Tomonaga *et al* 1986, Ellias *et al* 1988), and in co-operation with Epo to induce erythroid differentiation in the erythroleukemic cell line K562 (Tawhid *et al* 1989).

In addition to its effects on haemopoietic progenitor cells, GM-CSF also exerts a wide variety of actions on mature, fully differentiated neutrophils, eosinophils and macrophages. These actions can be either 1) direct effects, such as increasing cell survival (Begley *et al* 1986); or 2) indirect or so-called 'priming' effects that occur in

conjunction with a secondary stimulus, such as enhancement of neutrophil oxidative, phagocytic and cytolytic functions (Baldwin *et al* 1989), and priming of basophils for enhanced histamine release (Hirai *et al* 1988). As well as granulocyte-directed effects, GM-CSF also has a wide variety of macrophage directed functions, such as increased release of inflammatory mediators and growth factors (Oster *et al* 1992, Takamatsu *et al* 1996). These various effects on mature cells, are too numerous to discuss in full here, and the reader is refered to the excellent review by Baldwin (1992) for a full overview.

Many groups have attempted to examine the role of GM-CSF *in vivo*. One of the most interesting of these was that of Lang *et al* (1987), who overexpressed the GM-CSF gene in mice, under a retroviral promoter. These mice had a very unexpected compound phenotype, in that they were born with accumulations of macrophages in their eyes and striated muscle. In fact, the eyes were opaque, with extensive retinal damage that resulted in blindness. Many GM-CSF transgenic mice subsequently died after 2-4 months, with a chronic wasting syndrome. The ocular defects were attributed to autostimulation of macrophages, that are required for normal developmentally programmed tissue remodelling in the mouse eye (Cuthbertson *et al* 1989, Lang and Bishop 1993). Stanley *et al* (1994) later reported that GM-CSF was in fact dispensable for haemopoiesis, since GM-CSF deficient mice exhibited apparently normal haemopoiesis. These mice did however, develop an abnormal lung pathology that was characterised by infiltration of lymphocytes and large intraalveolar macrophages and which was reminiscent of the human disease, pulmonary alveolar proteinosis (PAP). Many GM-CSF deficient mice also developed inflammatory lung infections. A similar phenotype has been reported in mice deficient for the common component shared by the GM-CSF, IL-3 and IL-5 receptors, the βc component (Nishinakamura *et al* 1995). This phenotype is reversed following bone marrow transplantation, suggesting a haemopoietic origin (Nishinakamura *et al* 1996b). In fact, human PAP was recently reported to be associated with a defect in the GM-CSF/IL-3/IL-5 common chain (βc) (Dirksen *et al* 1997). Taken together, these reports suggest that GM-CSF is probably dispensible (or even redundant) for normal constitutive haemopoiesis, but that GM-CSF may be important for pro-inflammatory functions *in vivo*.

2.12 Stem Cell Factor (SCF)

SCF is a pleiotropic cytokine (reviewed in Broudy 1997), that functions primarily in the early stages of haemopoiesis. It was previously known as kit ligand, mast cell growth factor, or steel factor. The gene for SCF is encoded within the Steel or *Sl* locus (Zsebo *et al* 1990), whilst the gene for the SCF receptor, *c-kit*, is encoded by the white spotted or *W* locus (Huang *et al* 1990). A host of naturally occuring mutations at the *W* or *Sl* loci have been identified, and these have led to the identification and further characterisation of both SCF and its receptor. The absence of either SCF or Kit receptor results in death in utero, or perinatally as a result of severe anaemia (Besmer *et al* 1993). This, together with evidence from embryonic expression patterns for SCF (Orr-Urtrger *et al* 1990, Matsui *et al* 1990), suggests a developmental role for SCF.

SCF exists in two forms generated by alternative splicing of mRNA (Flanagan *et al* 1991); as a soluble form and also as a transmembrane form, which are both biologically active (Anderson *et al* 1990). Mice with deleted transmembrane and cytoplasmic domains (which results in only the soluble form of SCF) are viable but show several phenotypic abnormalities (Flanagan *et al* 1991, Brannan *et al* 1991), suggesting the transmembrane form does play a role *in vivo*. Recently, it was reported that overexpression of the membrane associated form of human SCF in mice (which in murine cells acts as an antagonist, through interference with the interaction between murine SCF and its receptor), resulted in impaired melanocyte development. Moreover, compound heterozygous mice that carried both a mutated *W* or *Sl* allele and the transgene for hSCF ($hSCF^{220}$) (i.e $hSCF^{220}$ / +; *W* /+ or $hSCF^{220}$ / +; *Sl* / +), showed a significantly reduced number of dermal mast cells, and defective thymocyte development (Kapur *et al* 1997). This study suggests that transmembrane SCF is required for the migration or survival/proliferation of melanocytes and dermal mast cells, and that c-kit may be involved in thymocyte differentiation. In fact, the ratio of soluble to transmembrane SCF varies considerably between different tissues (Huang *et al* 1992). In the bone marrow, stromal fibroblasts predominantly express the soluble form of SCF (Heinrich *et al* 1993a, Linenberger *et al* 1995). In addition to expression by fibroblasts, SCF is constitutively expressed by endothelial cells, another important cell type in the stromal microenvironment. Keratinocytes and gut epithelial cells have also been reported to express SCF (Longley *et al* 1993, Klimpel *et al* 1995) and SCF

mRNA has also been detected in enriched populations of stem cells and progenitor cells (Ratacjczak *et al* 1995). Whilst inflammatory stimuli such as IL-1 and TNF are strong inducers of many growth factors from the bone marrow (Broudy *et al* 1986), they are much less potent at stimulating the expression of SCF (Heinnich *et al* 1993a, Linenberger *et al* 1995).

SCF exhibits a wide variety of haemopoietic functions. It has been reported to accelerate the entry of haemopoietic stem cells into cell cycle (Leary *et al* 1992) and has been suggested to maintain the survival of long-term repopulating cells *in vivo* (Li and Johnson 1994). SCF, in conjunction with IL-3, also enhances the production of CFU-S cells (see section 1.3) *in vitro* (de Vries *et al* 1991). Addition of a blocking antibody to Kit receptor was shown to reduce the survival of CFU-S cells (Kodama *et al* 1992) but still allowed the survival of LTC-IC (see section 1.4.1), suggesting that SCF is dispensible for LTC-IC survival. Several studies have reported that SCF can act upon very primitive pre-CFU-C type cells, that give rise to colony forming cells. In addition, SCF in conjunction with IL-3 and other haemopoietic growth factors enhanced the number of BFU-E, CFU-GM and CFU-GEMM *in vitro* (Bernstein *et al* 1991, Migliaccio *et al* 1992). In fact, SCF now forms the basis for an increasing number of *ex vivo* expansion protocols (Brandt *et al* 1992), in conjunction with a number of different cytokines. In such protocols, enriched populations of primitive precursor cells are removed from the donor and the number of resulting progenitor cells is expanded *in vitro* with a cocktail of growth factors, before re-infusion. This can result in as much as a 200-fold expansion of BFU-E, CFU-GM, and CFU-GEMM from enriched haemopoietic stem cells (Brugger *et al* 1993). It is also suggested that SCF can promote haemopoietic progenitor cell survival, in the absence of cell division (Keller *et al* 1995).

In vitro, SCF has been reported to have many synergistic actions with other growth factors to support direct colony formation (McNiece *et al* 1991, Metcalf and Nicola 1991). SCF can synergise with factors, such as Tpo and IL-3, to promote the growth (but not differentiation) of megakaryocyte progenitors and cell lines (Broudy *et al* 1995).

SCF appears to be particularly important for mast cell production. The tissues of mice with mutations in the *Sl* locus (Sl/Sl^{d}) or W locus (W/W^{v}) contain less than 1 % of the normal numbers of mast cells (Kitamura *et al* 1978, 1979), although mast

cell progenitors have been identified in the bone marrow of these mice. The most primitive mast cell progenitor, the pro-mastocyte, proliferates and differentiates in response to SCF plus IL-3 (Rodewald *et al* 1996) and mast cells can be cultured in vitro from a variety of sources, using SCF in conjunction with IL-3 (Durand *et al* 1994).

Evidence also exists for a role for SCF in B and T lymphocyte development. SCF can synergise with IL-7 to stimulate proliferation of pre-B cells (Billips *et al* 1992), but it does not enhance the proliferation of the more primitive pro-B cells. (Faust et al 1993). Actions of SCF on NK and dendritic cell progenitors have also been documented (Shibuya *et al* 1995, Szabolcs *et al* 1995).

In addition to its many effects on haemopoietic stem and progenitor cells, SCF also exerts many functions on mature cells of the mast cell lineage and to a lesser extent, basophil lineage. For instance, SCF enhances secretion of mediators from mast cells, as well as their chemotaxis and adhesion (Columbo *et al* 1992, Dastych *et al* 1994) and also induces IgE-dependent histamine release from basophils (Columbo *et al* 1992).

SCF therefore, is a haemopoietic growth factor that acts primarily during the earlier stages of haemopoiesis, interacts with a variety of growth factors to regulate stem / progenitor cell proliferation and which is particularly important to cells of the mast cell lineage.

2.13 Leukemia Inhibitory Factor (LIF)

LIF is an unusual factor, that exerts its actions on haemopoietic and mature cells. LIF was characterised by several groups under various different names; such as differentiation inducing factor (DIF or D-factor), differentiation inhibitory activity (DIA), human interleukin for DA cells (HILDA) and cholinergic neuronal differentiation factor (CNDF), but these are now more commonly known as LIF (reviewed in Metcalf 1991). LIF shows sequence homology with OSM, IL-6, IL-11 and CNTF, and like LIF these all signal through the common gp130 subunit. LIF receptors are found on cells of the monocyte/macrophage lineage (Hilton *et al* 1991) and the number of LIF receptors was found to increase with maturation. LIF receptors are also found on megakaryocytes and on a subclass of T lymphocytes (Hilton *et al*

1991), as well as on osteoblasts (but not osteoclasts), fibroblasts, endothelial cells, adipocytes and embryonic stem cells (reviewed in Metcalf 1991).

LIF expression is normally almost undetectable. No expression can be detected in normal serum by bioassay, nor in tissues by northern blot analysis. One consistent exception to this, is the expression of LIF by metrial gland cells developing in the uterine wall at the site of blastocyst implantation (Croy *et al* 1991). LIF has however, been reported to be expressed by a variety of cell types following induction by a variety of stimulae, such as IL-1, TNF-α, and TGF-β (Wetzler *et al* 1990). Fibroblasts (Tomida *et al* 1984), T lymphocytes (Gearing *et al* 1987), monocytes and macrophages (Anegon *et al* 1990), stromal cells (Wetzler *et al* 1990) and astrocytes (Wesselingh *et al* 1990) have all been reported to express LIF.

The reported effects of LIF on haemopoietic stem cells *in vitro*, are relatively limited. LIF was originally reported not to have any effect on the survival or proliferation of adult haemopoietic cells (Metcalf *et al* 1988). It has however been reported to stimulate the proliferation of murine myeloid DA-1 cells (Moreau *et al* 1988) and also to promote the IL-3 induced proliferation of megakaryocytes and BFU-E cells (Leary *et al* 1990). The origin of the name, leukemia inhibitory factor, lies with the ability of LIF to suppress the self-renewal of clonogenic murine M1 leukemic cells (Metcalf *et al* 1988, Metcalf 1989). This suppression occurred in conjunction with the simultaneous differentiation of M1 cells by LIF. These cells differentiated into cells with macrophage morphology, expressed Fc receptors and exhibited other macrophage-like characteristics such as phagocytosis and an increased dependency upon M-CSF for their survival. (Metcalf 1989, Metcalf *et al* 1988). In addition to M1 cells, LIF can also suppress colony formation from HL-60 or U937 cells, but with negligible changes in differentiation (Maekawa *et al* 1989). More recent studies have however, suggested that LIF can in fact effect the survival of haemopoietic progenitor cells (Escary *et al* 1993). LIF deficient mice were generated, which showed dramatically decreased numbers of stem cells in the spleen and bone marrow. In the spleen, pluripotent stem cells (CFU-S), committed erythroid progenitors (BFU-E), and GM progenitors (GM-CFC) were all reduced by 70-90 %. In the bone marrow CFU-S numbers were decreased by 60 %, whilst the BFU-E and GM-CFC were less affected. This decrease in the CFU-S pool could be restored by addition of exogenous LIF. Primitive stem cells from LIF deficient mice were able to reconstitute the

haemopoietic systems of lethally irradiated normal mice, suggesting that LIF was not affecting the differentiation potential of stem cells and was instead required to maintain stem cell numbers. Female LIF deficient mice were also infertile, due to a defect in embryo implantation. It has since been reported, that LIF is able to directly synergise with growth factors to promote the proliferation of purified progenitor cells (Keller *et al* 1996). These reports support previous *in vivo* studies on LIF and suggest that the roles of LIF *in vivo*, may be more extensive than originally envisaged (Metcalf *et al* 1990).

The most striking function of LIF however, is perhaps its ability to prevent differentiation commitment of murine embryonic stem (ES) cells. ES cells are normally totipotent and will give rise to cells of all murine tissues when injected into a blastocyst, but in culture ES cells undergo differentiation and lose their totipotency. LIF was believed to be unique in its ability to block this predisposed differentiation, yet still maintain the totipotent abilities of ES cells (Williams *et al* 1988), but it is now evident that IL-6, IL-11, CNTF and OSM can all prevent ES cell differentiation (Conover *et al* 1993, Nichols *et al* 1994). LIF has proved of immense use in the development of ES cell based technologies, such as those employed in the generation of transgenic and knockout mice.

2.14 The Transforming Growth Factor Beta (TGF-β) Superfamily

The TGF-β superfamily consists of over 20 factors involved in the growth, differentiation and morphogenesis of cells from almost all lineages. This superfamily can be divided into several different sub-families; the TGF-β family, the activins and inhibins, Mullerian inhibitory substance and the DVR group that includes the bone morphogenetic proteins (reviewed in Massague 1990). The various members of this superfamily are structurally related to the prototypic TGF-β1 molecule, sharing a common precursor structure and conservation of seven or nine cysteine residues in the mature sequence. There is considerable variation at the level of sequence identity with members within a subfamily showing considerable sequence identity e.g. TGF-β1, β2 and β3 share a 64-82 % homology in their amino acid sequence, whilst the homology between members in different subfamilies is more disparate. As a full discussion of all

the members of the TGF-β superfamily is beyond the scope of this work, only the TGF-β subfamily will be discussed hereafter, with a particular emphasis on TGF-β1.

2.14.1 The TGF-β family

This subfamily consists of at least five members at present. Three distinct mammalian forms of TGF-β (TGF-β1, β2 and β3) have been identified from various species. Two other non-mammalian forms have also been reported, TGF-β4 in chickens (Jakowlew *et al* 1998) and TGF-β5 in Xenopus Laevis (Kondaiah *et al* 1990). The TGF-βs are amongst the most pleiotropic of cytokines, and their functions include proliferative and inhibitory effects upon cell growth and differentiation, wound repair and fibrosis, and inflammation. The most abundant form of TGF-β in mammals is TGF-β1.

2.14.2 Structure of TGF-β: TGF-β exists in a latent form

TGF-β1 is considered to be the prototypic form of TGF-β. The mature form of TGF-β1 is a 25 kD disulphide linked polypeptide, composed of two 12.5 kD homodimers. TGF-β1 is expressed as a precursor, 412 amino acids (412aa) in length (Derynck *et al* 1985), which then undergoes intracellular proteolytic cleavage. The precursor is composed of an N-terminal signal sequence, a pro-region, and a C-terminal region that contains the active domain. TGF-β is synthesised in the form of a latent complex which is inactive and unable to bind to TGF-β receptors (Pircher *et al* 1986). This latent TGF-β complex can exist in two forms, either as a small latent TGF-β complex or as large latent TGF-β complex (Wakefield *et al* 1988, and reviewed in Harpel *et al* 1992).

The active form of TGF-β1 consists of a disulphide-bonded dimer of the C-terminal 112aa of the TGF-β1 precursor polypeptide. In the small latent TGF-β1 complex, the active TGF-β1 dimer complex is non-covalently associated with a dimer of the amino-terminal remnant from the proteolytically cleaved precursor (the pro-region) (Gentry *et al* 1988, Wakefield *et al* 1989). This remnant is known as the latency associated peptide (LAP) and purified LAP can reassociate with mature TGF-β and inactivate it (Gentry *et al* 1987). *In vitro*, small latent TGF-β complex has been reported to be expressed in the culture medium of several cell types, including bone

cultures (Jennings and Mohan 1990, Bonewald *et al* 1991) and an erythroleukaemic cell line (Lioubin *et al* 1991).

In addition to the small latent complex, latent TGF-β also exists in the form of large latent TGF-β complex. Platelets have been shown to express this (Wakefield *et al* 1988), it has also been found in cell conditioned medium from several cell lines (Olofsson *et al* 1992, Lioubin *et al* 1991) and in cultures of smooth muscle and endothelial cells (Flaumenhaft *et al* 1993). This large latent TGF-β complex has been purified from platelets (Miyazono *et al* 1988) and is a 235 kD complex, composed of the small latent TGF-β complex (as described above) and an additional 125-160 kD protein called the latent TGF-β binding protein (LTBP). This LTBP is linked to the small latent TGF-β complex via disulphide bonds (Kanzaki *et al* 1990). The precise function of LTBP is unclear, although roles in assisting assembly and secretion of the large latent TGF-β complex (Miyazono *et al* 1991), activation of the latent TGF-β complex (Flaumenhaft *et al* 1993), assisting in the association of TGF-β to the ECM (Taipale *et al* 1994), or in stability (Brown *et al* 1990) have all been proposed.

The complex system that appears to have evolved to prevent TGF-β activation, strongly suggests that inappropriate expression of TGF-β is highly undesirable. However, the *in vivo* mechanisms by which the latent forms of TGF-β are converted to the mature, active form have yet to be fully elucidated. *In vitro*, latent TGF-β can be activated by a variety of chemical and physical treatments, including extreme changes of pH, heating, urea, or by the use of detergents (Lawrence *et al* 1985, Brown *et al* 1990). Many reports have also demonstrated that various enzymatic treatments will also activate latent TGF-β (Lyons *et al* 1988). Indeed proteolysis of latent TGF-β remains the most likely mechanism for activation and several mechanistic models have been proposed (reviewed in Harpel *et al* 1992). One of these proposed mechanisms suggests that the activation of TGF-β may involve the activation of plasminogen to plasmin. Apolipoprotein (a) inhibits the activation of plasminogen to plasmin and this was shown to result in the suppression of TGF-β activation (Grainger *et al* 1993). Further evidence for this came from the observation that the activation of TGF-β was inhibited in mice overexpressing the human apolipoprotein (a) gene (Grainger *et al* 1994).

One interesting twist to the functioning of TGF-β is the observation that almost all mature active TGF-β found in plasma, is associated with α_2-macroglobulin (α_2M) (O'Connor-McCourt and Wakefield 1987). α_2M is a highly abundant serum glycoprotein that is secreted by the liver and other cells, such as macrophages and which interacts with other cytokines (reviewed in James 1990). α_2M binds only to mature TGF-β and its function remains uncertain. Several mechanisms have been proposed, for example α_2M could simply function as a mechanism to remove excess TGF-β from the circulation (Lamarre *et al* 1991), alternatively it may actively scavenge active TGF-β.

2.14.3 Expression of TGF-β

TGF-β has been reported to be expressed by a very wide variety of cells in culture. The expression of the different mammalian isoforms of TGF-β varies according to the cell type. The widespread expression of TGF-β during many stages of embryonic development suggests an important role for TGF-β during development and morphogenesis (Lehnert and Akhurst 1988, Wilcox and Derynck 1988). Expression of TGF-β is also widespread in the adult system, with expression of TGF-β1, β2 or β3 mRNA detectable in most tissues and organs (Thompson *et al* 1989).

The predominant source of TGF-β is platelets (Assoian *et al* 1983), but a number of other mature haemopoietic cell types have also been shown to synthesise TGF-β, including activated macrophages (Assoian *et al* 1987) and B and T lymphocytes (Kehrl *et al* 1986a, 1986b).

2.14.4 The role of TGF-β as an immunoregulator

As indicated above, the expression of TGF-β appears widespread across the course of embryonic development. The production of TGF-β by mature immune cells such as macrophages suggests however, that TGF-β may also act as an immunological mediator. In fact, TGF-β is a very potent chemotactic agent for neutrophils (Brandes *et al* 1991, Reibman *et al* 1991) as well as for monocytes and macrophages (Wahl *et al* 1987), fibroblasts (Postelwaite *et al* 1987) and mast cells (Gruber *et al* 1994). The immunological functions of TGF-β appear to be mostly anti-inflammatory. TGF-β inhibits the activation of T cells and macrophages (Wahl *et al* 1988, Tsunawaki *et al*

1988) and inhibits the production of cytokines (Musso *et al* 1990), as well as inhibiting the production and secretion of IgM and IgG antibodies from B cells (Kehrl *et al* 1986a, 1991).

TGF-β has been reported to provide a protective effect in animal models of certain autoimmune diseases. Administration of TGF-β1 was shown to protect against collagen induced arthritis and experimental autoimmune encephalomyelitis (models for reactive arthritis and multiple sclerosis respectively) (Kuruvilla *et al* 1991, Racke *et al* 1991, Johns *et al* 1991). TGF-β has also been implicated in the impaired immune response associated with autoimmune deficiency syndrome (AIDS), where elevated TGF-β levels were observed in cultures of peripheral blood mononuclear cells derived from HIV-infected donors (Kekow *et al* 1990). In addition, TGF-β has also been suggested to suppress HIV expression and replication in infected monocyte/macrophages (Poli *et al* 1991), suggesting that TGF-β may be involved both in modulating viral infection and in facets of the immune dysfunction associated with AIDS.

The most compelling evidence for a direct role for TGF-β *in vivo* has come from studies in which mice were generated with a homozygous deletion of the TGF-β1 allele (TGF-β1$^{-/-}$) (Shull *et al* 1992, Kulkarni *et al* 1993). In these initial studies, approximately 50 % of TGF-β1$^{-/-}$ embryos experienced prenatal lethality, but the remainder survived and were born normally with no apparent developmental abnormalities. Subsequent reports have examined the reasons for the observed prenatal lethality and have suggested that TGFβ1 null embryos do in fact show some defects in haemopoiesis, as well as in embryonic vasculogenesis (Dickson *et al* 1995). The observed lethality may however, be dependent upon the genetic background (Dickson *et al* 1995). After approximately 2 weeks, perinatally surviving mice begin to develop a progressive wasting syndrome that results in death with 3-4 weeks of age. Histological analysis revealed that this wasting syndrome was accompanied by a multifocal, mixed inflammatory cell infiltrate and tissue necrosis. The infiltrate was observed in many organs including heart, lungs and liver. Comparative analysis of wild type and homozygous TGF-β1$^{-/-}$ mice revealed that several pro-inflammatory cytokines were upregulated, including TNF-α, IFN-γ and MIP-1α (Shull *et al* 1992). Furthermore, elevated levels of MHC class I and class II expression have also been

observed in TGF-β1$^{-/-}$ mice (Geiser *et al* 1993, Nakabayashi *et al* 1997). Recently, it was shown that treatment of TGF-β1$^{-/-}$ mice with the immunosuppressive agent rapamycin led to ablation of the wasting syndrome, gain of weight and an almost complete inhibition of inflammation (Borkowski *et al* 1996), supporting the notion that some of the defects observed in the compound phenotype of TGF-β$^{-/-}$ mice are attributed to an inflammatory role for TGF-β *in vivo*.

Although some mice do die in utero, the survival of the remainder initially suggested that TGF-β1 was at least partially dispensible for normal development, or was compensated for, by another isoform. However it is now clear that this postnatal survival is in fact due to maternal transfer of TGF-β1 (Letterio *et al* 1994). TGF-β1 can be detected in homozygous null mice derived from a heterozygous mother, but none can be detected in null mice derived from a homozygous null mother. Moreover, homozygous null pups born to homozygous null females (that were kept alive by administration of dexamethasone) died perinatally due to severe cardiac abnormalities, suggesting a role for TGF-β1 in cardiac development. Furthermore, the authors demonstrated that TGF-β1 was able to cross the placenta and was taken up by homozygous null foetal cells. Additional evidence for an *in vivo* role for TGF-β1 has arisen from the observation that TGF-β1$^{-/-}$ mice have a complete absence of epidermal Langerhans cells and suggests that TGF-β1 may also be involved in Langerhans cell generation (Borkowski *et al* 1996, 1997). Additional evidence that TGF-β may be involved in the development of Langerhans cell, is suggested by recent *in vitro* studies in which TGF-β1 promoted the development of dendritic cells from CD34+ progenitors, by blocking apoptosis (Strobl *et al* 1996, Riedl *et al* 1997).

2.14.5 TGF-β as a regulator of cell proliferation

Perhaps more study has been directed towards the role of TGF-β in the control of cell proliferation, than any other of its many functions. TGF-β can act either to inhibit or to stimulate cell proliferation, though it appears to mostly function as an inhibitor. In fact TGF-β is one of the most potent growth inhibitors identified to date and shows activity on a wide variety of both normal and transformed cell types. These include epithelial cells, endothelial cells, fibroblasts, keratinocytes, T and B lymphocytes, neuronal cells and haemopoietic progenitor cells (Tucker *et al* 1984,

Moses *et al* 1985, Roberts *et al* 1985, Shipley *et al* 1986, Kehrl *et al* 1986a, Kehrl *et al* 1986b, Ohta *et al* 1987). The degree of inhibition is highly variable between cell types, ranging from induction of apoptosis (Bursch *et al* 1993, Alam *et al* 1994a), to either almost complete growth arrest in lung epithelial cells (Tucker *et al* 1984) or a lengthening of the G1 phase of the cell cycle (reviewed in Massague *et al* 1992, or in Alexandrow and Moses 1995).

2.14.6 The role of TGF-β in haemopoiesis

TGF-β functions as a bidirectional regulator of haemopoiesis, inhibiting proliferation of primitive multipotential progenitors (Keller *et al* 1988, 1990) and yet also synergising with specific growth factors to enhance proliferation of more committed progenitors (Keller *et al* 1991). Autocrine production of TGF-β has been demonstrated to inhibit the growth factor-induced proliferation of haemopoietic progenitors (Hatzfeld *et al* 1991, Ploemacher *et al* 1993). TGF-β can induce the apoptosis of myeloid leukaemic cells, which can be reversed by certain haemopoietic growth factors (Lotem and Sachs 1992, Taetle *et al* 1993). TGF-β has also been reported to induce apoptosis of primitive murine haemopoietic progenitor cells (Jacobsen *et al* 1995b). This effect appears to be at least partially reversible, since a combination of IL-1, IL-6 and IL-11 reduced the negative effects of TGF-β and supported previous studies, demonstrating that TGF-β-induced inhibition can be overcome by a combination of growth factors (Jacobsen *et al* 1994c).

As mentioned above, TGF-β can also function as a stimulator of more committed progenitor cells. Keller *et al* (1991) showed that TGF-β stimulated GM-CSF-induced granulopoiesis by enhancing the proliferation and differentiation of progenitor cells. In general the effects of the three different mammalian isoforms of TGF-β on haemopoietic progenitors tend to be fairly similar. One reported exception is the study by Jacobsen *et al* (1991b) that showed TGF-β1 and -β2 were both able to bidirectionally modulate the proliferation of human progenitor cells, whilst TGF-β3 was unable to stimulate the proliferation of granulopoiesis (but it was a more potent inhibitor of human haemopoiesis than TGF-β1 or -β2).

It is now evident that the control of stem cell proliferation is regulated through a balance of both positive and negative regulators. Some regulators such as TGF-β

and MIP-1α appear to be able to function as both inhibitors and stimulators, depending upon the state of maturity and the growth factors present. Thus, adding yet another twist to understanding interactions occuring within the complex network of regulatory cytokines.

2.15 The Role of Growth Factors in the Regulatory Control of Haemopoiesis

Clearly it is impossible to discuss all the haemopoietic growth factors outlined in table 1.8.1, in detail. In addition to those already discussed, many others have more lineage-restricted effects such as erythropoietin on erythroid cells (reviewed in Krantz 1991), whilst the actions of other recently identified factors such as ftl3 ligand (Lyman *et al* 1993) and thrombopoietin (reviewed in Kaushansky 1995) are only just beginning to be understood. However, as outlined in the preceding sections of this chapter, growth factors and cytokines exert a wide variety of functions on cells of various stages of differentiation, from uncommitted progenitors through to mature terminally differentiated cell types. Whilst many of these have been strongly implicated in the control of haemopoiesis, their precise role remains highly controversial. At one extreme it has been proposed that these haemopoietic growth factors (HGFs) may only function as survival factors. At the other extreme, these HGFs have been suggested to actively instruct the haemopoietic differentiation program. One can summarise these mechanisms as being either stochastic or instructive, though there are clearly many variations on these two models. It is beyond the scope of this work to provide a detailed explanation of the various models for haemopoietic differentiation. Therefore, the following sections are merely meant to give a very brief summary of the potential role of HGFs in haemopoietic differentiation.

2.15.1 The stochastic model of differentiation

In this model, proposed in its original form by Till *et al* (1964), stem cells undergo self-renewal and differentiate in a random or stochastic manner. Ogawa and colleagues have proposed models involving either random restriction of a stem cell to a monopotential lineage, or through random lineage restriction of oligopotential progenitors (reviewed by Ogawa 1993). The role of HGFs within this stochastic model is proposed to be restricted to the survival and proliferation of stem cells and their

progenitors. Therefore in this model, the process of differentiation must occur through the action of intrinsic factors. Indeed, studies have shown that cytokines can prevent cells from apoptotic death and therefore act as "happiness factors" (Williams *et al* 1990). As also discussed previously in chapter 2, other studies have clearly implicated HGFs in the control of the cycling status of stem cells and their progenitors. One of the strongest pieces of evidence in support of a stochastic mechanism involving intrinsic control factors is that of Fairbairn *et al* (1993), using the IL-3 dependent murine FDCPmix cell line. These cells have the capacity to undergo differentiation along multiple myeloid lineages, but in the absence of IL-3 these cells undergo apoptosis. However, introduction of the gene for the anti-apoptotic factor bcl-2 into these cells, resulted in a significant delay in the time taken for the onset of apoptosis to occur. Moreover, this was accompanied by a spontaneous differentiation of cells along a variety of haemopoietic lineages. This suggests that in FDCPmix cells, IL-3 may act to maintain cell viability and to block differentiation. Recently it was reported that monocytes may have an intrinsic program of differentiation toward macrophages, that can occur in the absence of M-CSF (Lagasse and Weissman 1997). In this study *op/op* mice lacking functional M-CSF and exhibiting depressed macrophage levels, were crossed with mice engineered to overexpress Bcl-2 in the myeloid lineage. Suppression of monocyte apoptosis in serum-free and cytokine-free medium *in vitro* was sufficient to allow complete macrophage differentiation. In addition, this Bcl-2 overexpression also rescued diverse macrophage populations *in vivo* and resulted in a less severe form of osteopetrosis in these mice. This suggests that M-CSF acts to augment monocyte survival and allows them to respond to an intrinsic differentiation program.

2.15.2 The instructive or deterministic model of differentiation

In contrast to the above stochastic model, other researchers subscribe to an instructive or deterministic model (reviewed by Morrison *et al* 1997). In this model it is proposed that HGFs directly influence the differentiation program. Evidence in support of this model was eloquently provided by studies on the G-CSF receptor (G-CSF R). Dong *et al* (1994) reported the existence of a nonsense mutation in the G-CSF R, in patients with chronic neutropenia. The authors demonstrated that this mutation caused a truncation in a differentiation-promoting domain within the G-CSF R, resulting in the ablation of G-CSF induced differentiation but not G-CSF induced proliferation. Further

support for a direct effect of HGFs on differentiation (as opposed to indirect effects such as induction or promotion of cell survival, or proliferation of a progenitor cell after commitment) have been reported by Shah *et al* (1994, 1996). In these reports it was shown that glial growth factor or TGF-β superfamily members acted in an instructive manner, to restrict multipotent neural progenitors to a glial lineage. Johe *et al* (1996) have also reported the involvement of instructive mechanisms in the differentiation of CNS stem cells to astrocytes. Therefore strong evidence exists for instructive mechanisms, particularly in the differentiation of cells within the CNS.

2.16 Summary

Clearly the processes involved in the regulatory control of haemopoiesis are highly complex. As indicated in the above sections, various models have been proposed to explain the involvement of growth factors and a large amount of evidence exists to support each model. As to which model is correct, remains uncertain and in controversy? Indeed they may not be mutually exclusive, as it is theoretically feasible for aspects of both stochastic and instructive mechanisms to be involved in this regulation. However, regardless of whether haemopoiesis is the result of intrinsic factors operating in a stochastic manner or is the result of an instructive environment, an interacting network of cytokines and their receptors is implicated in the regulation of haemopoiesis. This network may or may not exhibit redundancy and clearly shows many overlapping actions, whilst others may be unique to individual cytokines. It is evident that the balance of actions between the different members of this network, is important for the survival, proliferation, differentiation and maturation of haemopoietic cells and thus the normal running of the immuno-haemopoietic system.

CHAPTER 3: MIP-1α and the Chemokine Superfamily

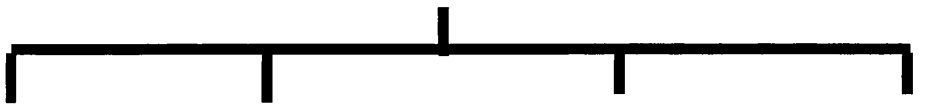
The role of MIP-1α as a haemopoietic stem cell regulator was first identified in 1990, when Graham *et al* (1990) purified a reversible inhibitor of CFU-A and CFU-S stem cell proliferation from the J774.2 murine macrophage cell line. They called this activity Stem Cell Inhibitor (SCI) and sequence analysis demonstrated that it was identical to a previously described molecule, macrophage inflammatory protein-1α (MIP-1α).

MIP-1α is a member of a rapidly expanding family of chemotactic cytokines, known as the chemokines. The biological functions of these chemokines are diverse and range from haemopoietic regulation to proinflammatory functions. The field has recently become a focus of attention and excitement, following the discovery of the critical role certain chemokines and their receptors play in the pathogenesis of HIV. An overview of chemokine biology (including recent advances), with a particular emphasis on the role of MIP-1α in the immuno-haemopoietic system follows.

3.1 The Chemokine Superfamily: Structure and Family Subdivision

As indicated above, MIP-1α belongs to the chemokine superfamily, which has expanded in a little under a decade from a handful of family members (Schall 1991, Oppenheim *et al* 1991) to now number in excess of 30-40 members (reviewed by Rollins 1997, Mackay 1997). In fact some researchers estimate that as many as 50-100 unique chemokines may eventually be identified. Table 3.1 lists the chemokines identified to date. The chemokines are all small inducible proteins, 8-18 kDa in size and are related to each on the basis of sequence homology and the presence of a characteristic cysteine motif in their N-terminus. Originally the family was subdivided into 2 classes, on the basis of the spacing of the first two N-terminal cysteine residues. Thus, in the "CC" or β subfamily these two cysteines are adjacent to each other, whilst in the "CXC" or α subfamily they are separated by a single residue. More recently, this designation has been complicated by the identification of unique members of two novel putative chemokine subfamilies. These are designated the C subfamily, with a sole member Lymphotactin (Kelner *et al* 1994), due to the presence of only a single N-terminal cysteine and the CX_3C subfamily with its sole member

Table 3.1 : The Chemokine Superfamily....so far



C-X-C	C-C	C	CX_3C
IL-8	MIP-1α	Lymphotactin	Fractalkine
Groα	MIP-1β		(=Neurotactin)
Groβ	RANTES		
Groγ	MCP-1		
NAP-2	MCP-2		
ENA-78	MCP-3		
GCP-2	MCP-4		
SDF-1	MCP-5		
PF-4	I-309		
IP-10	Eotaxin		
Mig	Eotaxin 2 (=MPIF-2)		
	HCC-1		
	TARC		
	MIP-3α (=LARC=Exodus)		
	MIP-3β (=ELC=Exodus-3)		
	DC-CK1 (=PARC)		
	MDC (=STCP-1)		
	MIP-3		
	MIP-1γ (=HCC-2=MIP-5=Lkn-1)		
	C-10 (=MRP-1)		
	MPIF-1 (=MRP-2=CCF18)		
	TECK (=TCA4)		
	6-Ckine (=Exodus-2=SLC)		

Fractalkine/Neurotactin (Bazan *et al* 1997, Pan *et al* 1997), in which the two amino terminal cysteines are separated by three amino acid residues. Members of each these four subfamilies, will be discussed in more detail in the following sections.

3.2 The CXC Chemokines

The CXC family of chemokines numbers at least 11 members to date (listed in table 3.1) and includes IL-8, PF-4, IP-10, Mig, the GRO proteins, NAP-2, ENA-78 and SDF-1. It is possible to further subdivide the CXC chemokines, based on the presence or absence of a so-called "ELR-motif", located in the N-terminal region just prior to the CXC motif. The ELR-chemokines such as IL-8, NAP-2, the GRO proteins, ENA-78, and GCP-2 are all potent neutrophil chemoattractants, whilst the non-ELR chemokines include PF-4, IP-10, Mig, and SDF-1 and show little or no neutrophil directed actions. Modification of these ELR residues prevents receptor binding and thus also neutrophil chemotaxis (Beall *et al* 1992, Clark-Lewis *et al* 1993) and insertion of an ELR motif into the N-terminus of non-ELR chemokines enables them to become neutrophil chemoattractants (Clark-Lewis *et al* 1993).

3.2.1 Interleukin-8 (IL-8)

IL-8 or neutrophil activating protein (NAP)-1 as it was originally called, is perhaps considered to be the prototypic CXC chemokine and is certainly the most extensively studied. To date, no rat or murine homologue of IL-8 has been identified. However, antibodies to human IL-8 inhibit lung inflammation in rats, suggesting a rodent IL-8 homologue does exist *in vivo*. Human IL-8 is produced by a wide variety of cell types, including monocytes, neutrophils, fibroblasts, endothelial cells, epithelial cells, and T lymphocytes and can be induced by a number of inflammatory stimuli such as IL-1, TNF, and LPS (reviewed by Stoekle and Barber 1990, Oppenheim *et al* 1991). IL-8 was originally identified as an activity that was directed towards neutrophils, but not monocytes (Yoshimura *et al* 1987, Shroeder *et al* 1987) and which may also chemoattract T lymphocytes (Larsen *et al* 1989). In addition to chemoattraction of neutrophils, IL-8 also induces degranulation and the respiratory burst in neutrophils (Walz *et al* 1987). IL-8 has also been suggested to play a role in angiogenesis (Koch *et al* 1992, Smith *et al* 1994a).

There is increasing evidence that IL-8 may also function as an inhibitor of haemopoietic stem cell proliferation. Broxmeyer *et al* (1993) showed that in addition to MIP-1α, other chemokines such as PF-4, IL-8 and MCP-1 can all function to suppress colony formation from GM-CSF plus SCF stimulated progenitor cells. Laterveer *et al* (1995, 1996a) reported that a single injection of IL-8 rapidly mobilised haemopoietic progenitors and that SCF augmented this mobilisation (Laterveer *et al* 1996b). Although no murine homologue of IL-8 has been identified to date, a putative murine IL-8 receptor homologue (mIL-8Rh) has, and mice have been generated with a homozygous null mutation in this gene (Cacalano *et al* 1994). These IL-8Rh$^{-/-}$ mice had an increased number of neutrophils, suggesting a negative role for the IL-8Rh in regulating neutrophil numbers. In addition, there was also an increased number of B cells in their lymph nodes, as well as enlarged spleens due to enhanced proliferation of specific myeloid elements and megakaryocytes. There also appeared to be enhanced myelopoiesis in the bone marrow. Thus, providing further evidence supporting a possible role for IL-8 or related chemokines in the regulation of haemopoiesis *in vivo*. Further evidence for the involvement of the IL-8Rh in the negative regulation of myeloid progenitor cells, was also reported in a more recent study (Broxmeyer *et al* 1996). This study compared normal mice and mice deficient in mIL-8Rh (mIL-8Rh$^{-/-}$) that were raised in either a normal or a germ-free environment. Myeloid progenitors from mIL-8Rh$^{-/-}$ mice were insensitive to inhibition by hIL-8 and MIP-2, a ligand for mIL-8Rh. A large expansion of myeloid progenitors was observed in these mice and this expansion was decreased in mice raised under germ-free conditions, suggesting that it was environmentally inducible.

3.2.2 Stromal Cell Derived Factor-1 (SDF-1)

The chemokine SDF-1 was originally cloned from a 'signal sequence trap library' (designed to enrich for proteins containing a signal peptide sequence) (Tashiro *et al* 1993). It was also cloned from a stromal cell line, as pre-B-cell growth-stimulating factor (PBSF) and as the name suggests, it stimulated the growth of B-cell progenitors *in vitro* (Nagasawa *et al* 1994). Unusually for a chemokine it is constitutively expressed in a broad range of tissues. The importance and apparent uniqueness of SDF-1 is only just beginning to emerge. Although SDF-1 is grouped with the CXC chemokines, it is equally distant/related to both CC and CXC

chemokines. Furthermore it has a unique degree of conservation with the murine and human homologues sharing 99 % amino acid identity, suggesting that of all the chemokines to date, SDF-1 is the least diverged from any prospective primordial ancestor.

An insight into the probable role of SDF-1 *in vivo* has come from the generation of mice in which the gene for SDF-1 has been disrupted (Nagasawa *et al* 1996a). These SDF-$1^{-/-}$ mice died perinatally, showing a severe reduction in the number of B cell progenitors in both the fetal liver and bone marrow and a reduction in myelopoietic progenitors in the bone marrow (but not the fetal liver), thus suggesting SDF-1 is a regulator of B cell lymphopoiesis and bone marrow myelopoiesis. These SDF-$1^{-/-}$ mice also exhibited a defect in the development of the cardiac ventricular septum. It has since been reported that SDF-1 is a low-potency, high efficacy lymphocyte chemoattractant *in vitro* (Bleul *et al* 1996a) and that it also chemoattracts monocytes, but not neutrophils (it is a non-ELR chemokine). Most recently, Aiuti *et al* (1997) demonstrated that SDF-1 was a chemoattractant for human $CD34^+$ progenitor cells. These progenitors consisted of both moderately primitive and more lineage-committed $CD34^+$ cells. This suggests that SDF-1 may be important in the migration and homing of bone marrow $CD34^+$ progenitors to different organs during development and to different niches during the differentiation and maturation of haemopoietic progenitor cells. Such a role certainly provides a feasible explanation for the myelopoietic defects observed in the SDF-$1^{-/-}$ mice. The role of SDF-1 in HIV pathogenesis is discussed in section 3.9.

3.2.3 IP-10 and Mig

Interferon-inducible Protein-10 (IP-10) was originally isolated in 1985, as an IFN-γ induced product from U937 cells (Luster *et al* 1985). IP-10 is expressed by a variety of cell types, including monocytes, keratinocytes, fibroblasts, endothelial cells and T lymphocytes (Luster *et al* 1985). IP-10 is a non-ELR chemokine and this is in keeping with its poor neutrophil activity (Dewald *et al* 1992), but it does appear to be chemotactic for monocytes (Taub *et al* 1996) and smooth muscle cells (Wang *et al* 1996). At the moment there is conflicting evidence as to whether IP-10 does, or does not chemoattract T lymphocytes (Taub *et al* 1996, Luster and Leder 1993, Roth *et al* 1995). In addition to chemotactic functions, IP-10 also appears to be an inhibitor of

angiogenesis (Angiolillo *et al* 1995), in contrast to IL-8 which appears to stimulate angiogenesis (Smith *et al* 1994a).

Monokine Induced by Interferon-γ (Mig) is a recently identified chemokine and like IP-10, it is also an IFN-γ inducible protein that was isolated from macrophages (Liao *et al* 1995). Mig is a chemoattractant *in vitro* for tumour infiltrating lymphocytes (TIL) and for peripheral blood lymphocytes (PBLs) (Liao *et al* 1995).

Mig and IP-10 can cross-desensitise each other in receptor activation (Liao *et al* 1995) and in fact, they appear to share the same receptor, CXCR3 (Loetscher *et al* 1996a). Like IL-8 (see section 3.2.1), both Mig and IP-10 have also been reported to act as inhibitors of haemopoietic progenitor cell proliferation (Sarris *et al* 1993, Sarris *et al* 1996, Schwartz *et al* 1997).

3.2.4 Other CXC Chemokines

The early study of PF-4 as an immunoregulator has been well documented (Stoekle *et al* 1990). PF-4 has also been reported to inhibit megakaryocytopoiesis (Gewirtz *et al* 1989, Han *et al* 1990, Gewirtz *et al* 1995) and more recently it was proposed that PF-4 (and IL-8 and NAP-2) supported the viability and survival of normal (but not leukaemic) progenitor cells (Han *et al* 1997). Little is known about the functions of other CXC chemokines like ENA-78 (Walz *et al* 1991) or some of the more recently identified members such as LIX (Smith and Herschman 1995) and granulocyte chemotactic protein-2 (GCP-2) (Proost *et al* 1993), beyond the initial reports of their isolation and characterisation.

3.3 The CC Chemokines

The number of CC chemokines appears to be much larger and their effects more diverse, than those of the CXC chemokine subfamily. Whilst many members were initially characterised as monocyte/macrophage chemoattractants, it is now evident that they act upon an extremely wide range of cell types, including basophils, mast cells, eosinophils, B and T lymphocytes and NK cells (reviewed by Rollins 1997). In general they do not exert neutrophil directed effects, although there are a few examples of specific chemokines that may be exceptions to this.

3.3.1 The Monocyte Chemotactic Proteins

3.3.1.1 Monocyte Chemotactic Protein-1 (MCP-1) / JE

Human monocyte chemotactic protein-1 or MCP-1 was first isolated in 1989 from monocytic cell lines and activated monocytes (Yoshimura *et al* 1989) and its amino acid sequence found to be identical to that of the previously cloned human homologue of the mouse JE gene (Rollins *et al* 1989). JE was originally reported as a gene that was highly inducible by platelet-derived growth factor (PDGF) in the 3T3 fibroblast cell line (Rollins *et al* 1988). It is now generally assumed that JE is the murine homologue of the human MCP-1 gene and JE is therefore often commonly also refered to as MCP-1 (although as discussed in section 3.3.1.3, recent evidence suggests this may not necessarily be the case).

MCP-1 is an extremely potent monocyte chemoattractant (Yoshimura *et al* 1989), but has also been reported to function *in vitro* as a chemoattractant for activated T lymphocytes (Carr *et al* 1994) and NK cells (Taub *et al* 1995a). MCP-1 is also a potent inducer of mast cell migration (but not degranulation) (Taub *et al* 1995b). In addition, MCP-1 has also been reported to induce IL-1 and IL-6 expression in monocytes (Jiang *et al* 1992). Interestingly, a very recent report suggested that MCP-1 may also be involved *in vivo* with other CXC and CC chemokines in neutrophil recruitment (but does not appear to do so on its own), suggesting the existence of a co-operative chemokine network *in vivo* (Tessier *et al* 1997). In an elegant study, Weber *et al* (1996) demonstrated that MCP-1 could be converted from a potent inducer of mediator release from basophils to an eosinophil chemoattractant, by deleting the N-terminal amino acid residue from full length mature MCP-1. They also demonstrated that deletion of another residue led to inactivity with both basophils and eosinophils, whilst deletion of three or four residues resulted in mutants that were active on both cell types. This strongly suggests that N-terminal processing is a potential mechanism for regulating MCP-1 cell selectivity and function. Interestingly, an antagonist of MCP-1 (9-76) derived by deletions in the N-terminus was recently shown to inhibit arthritis in the MRL-*lpr* model, a murine model in which mice spontaneously develop a chronic inflammatory arthritis similar to human RA (Gong *et al* 1997).

Several laboratories are currently trying to address the true *in vivo* role of MCP-1, through the generation and study of MCP-1 transgenic and "knockout" mice.

In one recent report the murine MCP-1 gene was coupled to the strong mouse mammary tumour virus (MMTV) promoter, resulting in mice (MMTV-MCP-1) with a high level of MCP-1 expression that corresponded to an increased susceptibility to intracellular pathogens (Rutledge *et al* 1995). No monocyte infiltrates were observed in any of these transgenic mice and this was proposed to be the result of monocyte unresponsiveness to locally produced MCP-1, due either to receptor desensitisation or disruption of monocyte chemoattractant gradients. In a second study by this same group, transgenic mice expressing MCP-1 under the control of the rat insulin promoter (RIP) were reported to elicit monocyte recruitment into pancreatic islets without the development of insulitis (diabetes) (Grewal *et al* 1997). When these RIP-MCP-1 transgenic mice were crossed with the MMTV-MCP-1 mice discussed above (thus generating mice expressing both transgenes), the resulting mice did develop a mild form of insulitis. This suggests that high systemic levels of MCP-1 prevent monocytes from responding to local MCP-1 and therefore the ability of MCP-1 to elicit monocyte infiltration will be dependent on its expression at lows levels and in it being restricted to the appropriate site.

There is increasing evidence that MCP-1 (and other chemokines) may be critically important mediators of inflammation in the central nervous system (CNS). Chemokine expression, particularly that of MCP-1 and MIP-1α, have been reported in many animal models of CNS inflammation (Berman *et al* 1996, reviewed by Ransohoff *et al* 1996).

3.3.1.2 MCP-2 and MCP-3

MCP-2 and MCP-3 are part of a small subgroup of CC chemokines with extensive sequence homology to MCP-1 (>60 %). In comparison to MCP-1 and MCP-3, MCP-2 action is still poorly understood. MCP-2 and MCP-3 were both identified through their monocyte chemoattractant abilities (Van Damme *et al* 1992) and they are both as effective as MCP-1 in this respect. Like MCP-1, they have both been reported to function as T lymphocyte chemoattractants. It appears that the biological effects of MCP-2 and MCP-3 partially overlap with those of MCP-1, yet others are clearly distinct. They often appear however, to be less effective and are produced at lower levels than MCP-1 (reviewed by Proost *et al* 1996). Whilst all three MCPs chemoattract basophils, MCP-3 is a particularly effective stimulator of basophil

chemotaxis and release (Alam *et al* 1994b). The putative murine homologue of human MCP-3 has been identified (Minty *et al* 1993), and was originally cloned as MARC from an IgE plus antigen stimulated mast cell line (Kulmburg *et al* 1992) and subsequently from 3T3 fibroblasts as fibroblast induced cytokine (*fic*) (Heinrich *et al* 1993b).

3.3.1.3 MCP-4 and MCP-5

MCP-4 and MCP-5 are the two most recently described MCPs. MCP-4 shares closest homology to MCP-3 and another CC chemokine, eotaxin. In fact MCP-4 appears to share similar functional properties to MCP-3 and eotaxin. Like MCP-3 and eotaxin it is also a strong chemoattractant for eosinophils, as well as for monocytes and T lymphocytes (Uguccioni *et al* 1996) and it is also reported to stimulate histamine release from basophils (Garcia-Zepeda *et al* 1996).

To date, only murine MCP-5 has been identified (Jia *et al* 1996, Sarafi *et al* 1997). MCP-5 appears to share greatest homology with human MCP-1 (66 % amino acid identity) and is also structurally very similar too. In fact this similarity is even greater than that shown between human MCP-1 and the supposed murine homologue, JE (55 % identity). This suggests that JE may not in fact be the murine homologue of human MCP-1, although Jia *et al* (1996) did find evidence for a potential human MCP-5 gene that was not MCP-1.

3.3.2 RANTES

RANTES (**R**egulated on **A**ctivation **N**ormal **T**-cell **E**xpressed and **S**ecreted) was identified by Schall *et al* in 1988, and found to be a gene inducible by antigenic or mitogenic stimulation in a variety of T cell lines and circulating T lymphocytes (Schall *et al* 1988). RANTES is produced by a variety of cell types including platelets (Kameyoshi *et al* 1992) and eosinophils (Lim *et al* 1996). RANTES is a potent chemoattractant of monocytes and subsets of T lymphocytes, particularly of the $CD4^+$, $CD45RO^+$ memory phenotype (Schall *et al* 1990), but is also a very potent chemoattractant of $CD8^+$ T cells (Roth *et al* 1993). RANTES has also been reported to chemoattract and activate eosinophils (Rot *et al* 1992) and basophils (Bischoff *et al* 1993), as well as NK cells (Taub *et al* 1995a).

3.3.3 Other CC Chemokines

There are now over twenty known, distinct CC chemokines, many only recently discovered and no doubt others remain to be identified still. Briefly, others include Eotaxin and Eotaxin-2 (also known as MPIF-2), which are potent chemoattractants and activators of only eosinophils and basophils (Jose *et al* 1994, Forssmann *et al* 1997). I-309 and its putative murine homologue TCA-3 (Miller *et al* 1989, Burd *et al* 1987) are produced by activated mast cells and T cells and are unusual in having an extra pair of cysteines. TCA-3 is activated on a wide variety of cell types, including neutrophils. Other novel chemokines include C10 (Orlofsky *et al* 1991), MIP-1γ (Mohamadzadeh *et al* 1996), CCF18 or MIP-related protein-2 (MRP-2) (Youn *et al* 1995, Hara *et al* 1995). Recently two laboratories isolated a novel chemokine with an extended C-terminus that includes two extra cysteines, known as 6C-Kine (Hedrick and Zlotnick 1997) or Exodus-2 (Hromas *et al* 1997a). Another interesting recent discovery, was that of a novel chemokine known as thymus expressed chemokine or TECK, that appears to be specifically expressed by thymic dendritic cells and which may be involved in T cell development (Vicari *et al* 1997). In most cases the functions of these novel chemokines are unknown.

3.4 The C Subfamily: Lymphotactin

To date Lymphotactin (Lptn) is the only member of the so called C subfamily. It only has 2 cysteines, which correspond to cysteines 2 and 4 in the CXC and CC chemokines. Lptn was originally cloned from activated pro-T cells (Kelner *et al* 1994) and the human homologue, SCM-1, was subsequently isolated (Yoshida *et al* 1995). Lptn was shown initially to attract T lymphocytes, particularly of type $CD4^+$ and $CD8^+$, as well as thymocytes (with the exception of $CD4^+CD8^+$ thymocytes) (Kelner *et al* 1994). More recently, Lptn has also been reported to chemoattract NK cells both *in vitro* and *in vivo* (Hedrick *et al* 1997). In addition to pro-T cells and activated T cells, Lptn is now known to be produced by NK cells (Hedrick *et al* 1997) and IgE plus antigen activated mast cells (Rumsaeng *et al* 1997).

3.5 The CX_3C Subfamily: Fractalkine / Neurotactin

The recent identification of a putative fourth chemokine subfamily has caused considerable interest. This is because fractalkine, the only member identified to date,

appears unique in being the only known membrane-anchored chemokine. Fractalkine was cloned by Bazan *et al* (1997) from non-haemopoietic cells and shown to be a 373-amino acid glycoprotein that carried a chemokine domain on a long extended mucin-like stalk. Fractalkine exists either in a membrane bound form or as a secreted protein. The secreted form attracts T cells and monocytes, whilst the anchored form promotes leukocyte adhesion. The expression of fractalkine is also unique amongst chemokines, with little or no expression detectable in peripheral blood leukocytes. Fractalkine can however, be strongly induced by IL-1 and TNF. Fractalkine was also cloned independently as neurotactin and shown to be predominantly expressed in mouse brain, with elevated expression in mice with EAE or in LPS treated mice (Pan *et al* 1997). However, in contradiction to Bazan *et al*, neurotactin was reported to attract neutrophils. The precise function of fractalkine / neurotactin is still uncertain, but it may well be involved in leukocyte adhesion and CNS inflammation.

3.6 MIP-1α

MIP-1α and MIP-1β are clearly distinct molecules. However, they do share some striking similarities in their functions, barring a few specific exceptions. Therefore, the following discussion is largely limited to that of MIP-1α, unless stated otherwise.

3.6.1 Characterisation of MIP-1α

MIP-1α and MIP-1β were originally isolated as MIP-1 from the macrophage cell line RAW264.7, stimulated by LPS (Wolpe *et al* 1988). MIP-1 ran as a doublet composed of two peptides with very similar molecular weights of ~8 kDa. This doublet was subsequently found to comprise two distinct proteins, which were renamed MIP-1α and MIP-1β (Sherry *et al* 1988). The cDNA previously cloned as MIP-1 corresponded to that of MIP-1α (Davatelis *et al* 1988). The amino acid sequences of MIP-1α and MIP-1β are 59.8 % identical, whilst their cDNAs share 56.7 % identity. The human homologues of MIP-1α and MIP-1β had previously been identified as LD78 (Obaru *et al* 1986) and Act-2 (Lipes *et al* 1988) respectively. The MIP-1 proteins are encoded for by distinct genes and clustered on chromosome 17 in humans, and on chromosome 11 in mice, along with most other CC chemokines.

One of the original observations made during the isolation of MIP-1 was its propensity to undergo self-aggregation (Wolpe *et al* 1988). In fact this self aggregation is a phenomenon noted for both human and murine MIP-1α and MIP-1β. The aggregation results from non-covalent and electrostatic interactions between molecules and occurs to such an extent that multimers may result with molecular masses in excess of 100 kDa in physiological buffers *in vitro* (Graham *et al* 1992) and even greater aggregation occurs at higher concentrations (>10^6 kDa). Further understanding of this aggregation has been achieved through the derivation of three MIP-1α mutants, in which the C-terminal acidic amino acids have been sequentially neutralised, resulting in the generation of non-aggregating forms of MIP-1α (Graham *et al* 1994). These three mutants formed tetramers, dimers and monomers respectively, which were equipotent to each other and to wild type MIP-1α, in both stem cell inhibition assays and monocyte shape-change assays. This suggested that aggregation was not necessary for the function of MIP-1α in these assays and implied that the aggregated forms spontaneously disaggregate and are ultimately functional as monomers, as has been confirmed in more recent studies. However, it should be noted that these results are in contrast to those obtained by Mantel *et al* (1993) in a previous study. In this study, it was proposed that the inhibitory activity of aggregated MIP-1α resided in small amounts of monomeric MIP-1α in the aggregated preparations and it was further suggested, that the monomeric MIP-1α therefore had 1000-fold higher activity per unit weight than the aggregated forms. Graham *et al* (1994) reported however, that the aggregated form (separated from any monomeric MIP-1α) was as active as the monomeric form and therefore concluded that the aggregated form spontaneously disaggregated under the assay conditions used.

Recently, the actions of stem cell inhibition and monocyte chemoattraction have been uncoupled. A MIP-1α mutant has been generated in which the proteoglycan binding domain had been disrupted by mutagenesis of two basic amino acids within this site (Graham *et al* 1996). This mutant (HepMut) was unable to bind proteoglycans or bind to the human or murine MIP-1α receptor, CCR1, and was also severely compromised in its ability to function in the monocyte shape change assay. In contrast however, HepMut was still fully active in both human and murine stem cell inhibition assays. Therefore, it appears that the proteoglycan binding region mutated in this study

is critical for binding of MIP-1α to CCR1, but not for inhibition of stem cell proliferation and suggests, that inhibition may occur through another receptor distinct from CCR1.

3.6.2 Normal Expression of MIP-1α

MIP-1α mRNA has been reported to be expressed by a wide variety of haemopoietic cell-types, including monocytes and macrophages (Davatelis *et al* 1988), T lymphocytes (Obaru *et al* 1986), mast cells (Burd *et al* 1989), eosinophils (Costa *et al* 1993), basophils (Li *et al* 1996), neutrophils (Kasama *et al* 1993), NK cells (Bluman *et al* 1996) and epidermal langerhans cells (Heufler *et al* 1992). In addition, several non-haemopoietic cell-types such as fibroblasts (Nakao *et al* 1990) and microglial cells (Hayashi *et al* 1995) have also been reported to express MIP-1α.

MIP-1α has been reported to be an inhibitor of haemopoietic stem cell proliferation (Graham *et al* 1990) and although expression of MIP-1α mRNA is detectable by PCR based techniques in normal bone marrow, the levels of MIP-1α are low. This is a situation which is in fact common for other haemopoietic cytokines (see section 1.6.1). One explanation for these low levels, is that MIP-1α protein is sequestered and held in a local microenvironment through interactions with proteoglycans and therefore only a low level of expression is necessary for an effect within a localised microenvironment. Although as indicated above, such proteoglycan interaction is not apparently necessary for stem cell inhibition. Another explanation, is that under normal circumstances the expression of MIP-1α is held in check and is only released under conditions of stress, such as inflammation. One possible candidate for the *in vivo* suppression of MIP-1α, is TGF-β. Reports from our laboratory (Maltman *et al* 1993, 1996) have defined an endogenous reciprocal relationship in bone marrow derived macrophages between MIP-1α and TGF-β, whereby elevation of MIP-1α will lead to the increased expression of TGF-β, which in turn will result in a suppression of MIP-1α expression. Thus, in the bone marrow, where levels of TGF-β are readily detectable, TGF-β would act as the predominant partner and keep the expression of MIP-1α in check. Outside of the bone marrow, several other cytokines have also been shown to downregulate the expression of MIP-1α, including IL-10 (Kasama *et al* 1994) and IL-4 (Standiford *et al* 1993a).

MIP-1α was originally identified as an LPS-inducible gene in macrophages, but a number of other stimuli also induce MIP-1α expression. These include IgE in mast cells (Burd *et al* 1989) and IL-1, IL-2, and IL-6 in monocytes (Maciejewski *et al* 1992). Several studies have tried to address the transcriptional elements involved in inducible MIP-1α expression, but with limited success. Comparative analysis of the promoters for murine MIP-1α and β revealed a conserved CK-1 element (Widmer *et al* 1993) previously identified in the murine GM-CSF gene (Stanley *et al 1985)*, whilst Ritter *et al* (1995) demonstrated that a novel transcription factor named MIP-1α nuclear protein (MNP) was required for transcription of human MIP-1α. Analysis of LPS induction has revealed that members of the C/EBP, NF-KB, and c-ets transcription factor families may be involved in LPS induced MIP-1α transcription (Grove and Plumb 1993). Another report, by Proffitt *et al* (1995), suggested that ATF/CREB and AP-1 related proteins may be involved in regulating MIP-1β expression following LPS induction, implying that whilst MIP-1α and MIP-1β are co-expressed in many cell types they may exhibit differential transcription under certain circumstances.

3.6.3 Expression in Disease and Inflammation

Elevated MIP-1α expression has been observed in a number of disorders and disease states. For instance, a significant increase in MIP-1α mRNA expression was observed in bone marrow nucleated cells derived from patients with aplastic anaemia (AA) and myelodysplastic syndrome (MDS) (Maciejewski *et al* 1992). It is probable that this aberrant expression results from dysregulated expression of other cytokines within the bone marrow as a result of the disease state, though it is also possible that aberrant MIP-1α expression might play an etiological role in the development of AA and MDS.

Increased expression of MIP-1α has been observed in both human interstitial lung disease (Standiford *et al* 1993b) and also in a murine model of interstitial lung disease (Smith *et al* 1994b), as well as in several other animal models of human diseases such as murine endotoxemia (a model for sepsis) (Standiford *et al* 1995).

MIP-1α expression is also elevated in TGF-$\beta^{-/-}$ mice (Shull *et al* 1992). This finding is certainly in keeping with the known endogenous reciprocal relationship with

TGF-β (see previous section) and suggests that this interaction may indeed occur *in vivo*. It is not clear however, whether MIP-1α is a direct mediator of the multifocal inflammation observed in these mice or if it is merely a secondary consequence of it.

In addition to classical inflammatory disease, MIP-1α has also been implicated in several auto-immune diseases, most notably in rheumatoid arthritis (RA), as well as in inflammatory neurological disease. RA is characterised by elicitation and activation of various leukocyte populations within the synovial space and in joint tissue. These recruited leukocytes are known to be critical participants in the resulting pathology. A number of chemokines have been implicated in the recruitment of leukocytes in RA (reviewed by Kunkel *et al* 1996), one of these being MIP-1α (Koch *et al* 1994). Aberrant MIP-1α expression has also been documented by many researchers in a variety of inflammatory neurological disease states. One of the most intensively studied areas has been that of multiple sclerosis (MS), characterised by focal T cell and macrophage infiltration into white matter and resulting demyelination. Significant elevation of MIP-1α has been reported in MS patients undergoing relapse (Miyagishi *et al* 1995). Experimental allergic encephalomyelitis (EAE) is an experimental model frequently used to study MS. Investigation of EAE, has revealed that the kinetics of MIP-1α expression (and certain other chemokines) parallels that of leukocyte recruitment and disease severity (Miyagishi *et al* 1997). MIP-1α has been reported to be produced by microglial cells within the central nervous system (CNS) (Hayashi *et al* 1995) and by glial and neuronal cells within the brain (Ishizuka *et al* 1997). Perhaps most conclusive, is the report by Karpus *et al* (1995), in which they demonstrated that administration of anti-MIP-1α antibodies prevented the development of EAE. Taken together these studies strongly implicate MIP-1α (and other chemokines) as important mediators of inflammation within the CNS.

3.6.4 Pro-inflammatory Functions of MIP-1α

During the initial characterisation of MIP-1α, as MIP-1, Wolpe *et al* (1988) reported that injection of MIP-1 into the footpads of mice resulted in inflammation, characterised primarily by a neutrophil infiltrate. MIP-1 was also shown to attract neutrophils and induce a respiratory burst *in vitro*. Since that initial report the precise relationship between MIP-1α and neutrophils has remained confused. Firstly, the

report of Wolpe *et al* (1988) used a supraphysiological dose of MIP-1. A later report by McColl *et al* (1993) suggested that human MIP-1α had only very minimal effects on neutrophil attraction and activation. In contrast however, was a later report that murine MIP-1α was a mediator of neutrophil recruitment in murine endotoxemia (Standiford *et al* 1995). One possible explanation is that MIP-1α does not play a role in neutrophil function in humans, but that it does in rodents. In support of this is the observation that rodent, but not human, neutrophils express abundant CCR1, a MIP-1α receptor. In addition, IL-8 which is a potent neutrophil chemoattractant has not been identified in rodents, nor has its receptor CXCR1 been detected on rodent neutrophils. It is possible therefore that in rodents, MIP-1α and CCR1 can fulfil the roles played by IL-8 and CXCR1 in humans. This notion is however, difficult to reconcile with the identification of a putative murine IL-8Rh that has been implicated in neutrophil function (Cacalano *et al* 1994). At present the role of MIP-1α in neutrophil function is unclear, but it does underline the fact that caution that should be used in assuming that there is a direct correlation between human and murine chemokines, their receptors, and their functions.

MIP-1α is unquestionably a potent monocyte/macrophage chemoattractant (Standiford *et al* 1993b), but in addition it is also involved in regulating the effects of many other immune cell types. Macrophages are not only a predominant source of MIP-1α expression, they are also themselves targets for the action of MIP-1α. Fahey *et al* (1992) reported that native MIP-1 purified from LPS stimulated macrophages (containing both MIP-1α and MIP-1β), was able to enhance the antibody independent macrophage cytotoxicity for tumour cells and stimulate the proliferation of mature tissue macrophages. In addition MIP-1α, but not MIP-1β, was able to induce secretion of TNF, IL-1α and IL-6. An excess of MIP-1β was in fact able to suppress the MIP-1α induced production of TNF.

It is clear, that whilst MIP-1α and MIP-1β appear to be co-expressed from many cell types and show many similar functions, they do also exert some distinct ones too. This is most apparent in their abilities to regulate stem cell proliferation (see below), but also in their effects on different lymphocyte populations. Taub *et al* (1993) showed that human MIP-1α preferentially attracted activated $CD4^+$ T cells, whilst human MIP-1β was preferential for activated $CD8^+$ T cells. This differential attraction

was confirmed by Schall *et al* (1993), who also demonstrated that MIP-1α was a more potent B cell chemoattractant than MIP-1β. Although proteoglycan binding appears dispensable for the function of MIP-1α in stem cell assays, a report by Tanaka *et al* (1993) suggests that proteoglycan binding may be important for inflammatory function. MIP-1β was shown to be present on lymph node endothelium and this proteoglycan bound MIP-1β induced the binding of T cell to VCAM-1 *in vitro*. This supports the notion that proteoglycans are involved in the localisation of chemokines to endothelial and other cell surfaces, where they act to recruit immune cells from the periphery to sites of inflammation.

In addition to actions on monocytes, T cells and neutrophils, MIP-1α has also been reported to activate basophils (Alam *et al* 1992), chemoattract (but not degranulate) IgE plus antigen activated mast cells (Taub *et al* 1995b) and induce the migration and activation of eosinophils (Rot *et al* 1992). MIP-1α has also been reported to enhance IgE and IgG4 production from human B cells (Kimata *et al* 1996) and to up-regulate synthesis of the latent metalloproteinase, pro-metalloproteinase-9 (proMMP-9) from lymphocytes (Johnatty *et al* 1997). Taken together, these reports demonstrate an important role for MIP-1α in the recruitment of specific immune cell-types into various tissues during inflammation. In addition MIP-1α appears to play a modulatory role on a variety of functions exerted by these recruited cells. MIP-1α also plays a key role in the inflammatory cascade, through its ability to induce production of other cytokines such as TNF and IL-1, that may in turn induce additional MIP-1α production and thus further amplify the local inflammatory response.

The most conclusive evidence verifying the *in vivo* importance of MIP-1α has come from the recent generation of MIP-1α "knockout" or "null" mice (MIP-1α$^{-/-}$), in which the MIP-1α gene was disrupted (Cook *et al* 1995). These mice had no overt haemopoietic abnormalities, suggesting that either MIP-1α is dispensable for normal haemopoiesis or is functionally redundant and its actions are replaced by another factor in these MIP-1α$^{-/-}$ mice. In contrast however, MIP-1α was required for generation of an inflammatory response to viral infection. Homozygous null mice were resistant to coxsackievirus-induced myocarditis compared with infected wild type mice, which did develop myocarditis. A reduced pneumonitis and delayed viral clearance was also observed in influenza virus infected MIP-1α$^{-/-}$ mice compared with

infected wild type mice. Thus, MIP-1α is involved in the inflammation associated with viral infection *in vivo*.

3.6.5 The Role of MIP-1α as a Haemopoietic Regulator

The first report on the ability of MIP-1α to function as a regulator of haemopoietic stem cell proliferation, was by Broxmeyer *et al* (1989). They used purified MIP-1, containing both MIP-1α and MIP-1β, and observed that addition of MIP-1 led to enhanced proliferation of M-CSF or GM-CSF stimulated committed progenitors (GM-CFC). MIP-1 had no effect on G-CSF stimulated colony formation. Subsequently, Graham *et al* (1990) purified the previously identified stem cell inhibitor (SCI) or NBME-IV activity (Lord *et al* 1976) and discovered that it was identical to MIP-1α. They were able to demonstrate that SCI/MIP-1α inhibited colony formation from CFU-A type stem cells and its *in vivo* correlate, CFU-S, but not that of more committed GM-CFC progenitors. Neutralising polyclonal serum raised against MIP-1 was also able to block the inhibitory activity of both SCI and recombinant MIP-1α. MIP-1β however, was unable to inhibit stem cell proliferation, at the concentrations used in these studies. Broxmeyer *et al* (1990) subsequently confirmed the inhibitory action of MIP-1α and the inability of MIP-1β to function as an inhibitor. This group also reported that MIP-1β was in fact able to block the suppressive effects of MIP-1α on BFU-E, CFU-GEMM and CFU-GM colony formation (Broxmeyer *et al* 1991). However, this block only occurred when MIP-1β was added in an excess to MIP-1α, as a 1:1 ratio did not result in a block of MIP-1α inhibition (explaining why MIP-1, composed of a 1:1 ratio of MIP-1α and β, was active as an inhibitor in the earlier study by Broxmeyer et al 1990).

The ability of MIP-1α to function *in vivo* as an inhibitor of stem cell proliferation has also been addressed in several studies. Dunlop *et al* (1992) demonstrated that human MIP-1α was active in a dose-dependent manner *in vitro* on d12 CFU-S cells, and to a lesser extent on more mature d8 CFU-S cells. In the same study, the authors reported that administration of multiple doses of MIP-1α was sufficient to protect the CFU-A / S compartment, following treatment with the cytotoxic drug cytosine arabinoside (Ara-C). Lord *et al* (1992) have reported that MIP-1α will also protect CFU-S cells against the cytotoxic effects of hydroxyurea

(HU). However, a later study by Quesniaux *et al* (1993), showed that more primitive stem cells were not protected by human MIP-1α. In this model, a primary injection of 5-FU was used to eliminate late progenitors and cause the more primitive, and normally quiescent, LTR stem cells (see section 1.3) to enter the cell cycle and therefore become sensitive to administration of a second dose of 5-FU. MIP-1α did not prevent the depletion of LTR stem cells following the second 5-FU injection, suggesting that it did not prevent cycling of the LTR stem cells. MIP-1α was however, able to inhibit the formation of more mature haemopoietic progenitors (GM-CFC, BFU-E, $CFC\text{-}_{multi}$, and pre-CFC_{multi}). It should be noted that cytotoxic drugs (such as Ara-C, HU, and 5-FU) that target specific stages of the cell cycle, are not the only drugs utilised in chemotherapy. They are often used in combination with non-S phase drugs, such as cyclophosphamide. Studies such as those recently reported by Marshall *et al* (1997), are currently being undertaken to address whether or not MIP-1α is a potentially useful myeloprotective agent against therapeutic regimes that are more applicable to current clinical settings.

The therapeutic value of MIP-1α against leukaemic cells has also been investigated. Eaves *et al* (1993) have demonstrated that MIP-1α does not inhibit the cycling of primitive chronic myeloid leukaemia (CML) cells supported in long-term cultures (LTC). MIP-1α did however, inhibit the activation of normal primitive progenitors (but not more mature ones) in similar LTCs. This provides hope that MIP-1α could prove useful in a therapeutic role, by providing protection of normal primitive stem cells against any cytotoxic agents, such as those used in chemotherapy, whilst simultaneously leaving CML cells susceptible. Chasty *et al* (1995) also reported that CML cells were relatively refractory to inhibition by MIP-1α. Moreover, the authors demonstrated that these CML cells expressed similar levels of MIP-1α receptors as did normal $CD34^+$ cells. This suggests the inability of MIP-1α to inhibit CML cells may lie in a downstream signalling event. Interestingly, Ferrajoli *et al* (1994) reported that MIP-1α could inhibit the proliferation of subsets of acute myelogenous leukaemia (AML) progenitors. However, subsequently Owen-Lynch *et al* (1996) have shown that sub-sets of AML cells are not inhibited by MIP-1α. In this study, autonomous AML blast cells were refractory to MIP-1α inhibition, whilst cytokine responsive AML cells were inhibited by MIP-1α in the presence of either

GM-CSF or SCF. However, when GM-CSF and SCF were used in combination, only a small number were inhibited by MIP-1α with the remainder remaining refractory. Interestingly, in the earlier study reported by Ferrajoli *et al* (1994) only single cytokine additions were used. In contrast, the later study of Owen-Lynch *et al* (1996) used either single cytokine additions (in which the CML cells were inhibitable), or a combination of cytokines (in which most samples were refractory to MIP-1α inhibition), or no cytokine treatment to allow autonomous growth. The use of multiple cytokines is more likely to be representative of the environment *in vivo,* and therefore this study suggests that in addition to CML cells, autonomous AML cells and some subsets of cytokine responsive AML cells may be potential targets for therapeutic regimes, in conjunction with MIP-1α as a protective agent.

In addition to the inhibition of bone marrow haemopoiesis, MIP-1α also functions *in vitro* as a potent, reversible inhibitor of both human and murine epidermal keratinocyte proliferation (Parkinson *et al* 1993). MIP-1α mRNA was previously shown to be produced by epidermal langerhans cells (Heufler *et al* 1992) and it has been proposed, that within the skin, epidermal stem cells are held quiescent by MIP-1α derived from langerhans cells located within the same epidermal proliferative unit (Graham and Pragnell 1992b).

Inhibition by MIP-1α, seems to occur on a relatively restricted subset of haemopoietic stem cells, represented by the CFU-A / S compartment. MIP-1α will not inhibit the most primitive stem cells, nor more mature ones. In fact it appears to function as a stimulator of more mature progenitors. In this respect, MIP-1α shares many similarities with TGF-β. Indeed Keller *et al* (1994) have shown that MIP-1α and TGF-β show overlapping, as well as distinct functions on haemopoietic stem cells. MIP-1α and MIP-1β have extensive homology to each other, but MIP-1α is more effective as an inhibitor and MIP-β may play a role in blocking this inhibition. It is also becoming evident that many other chemokines may function as inhibitors of haemopoiesis. A number of these novel chemokines have been reported to act as haemopoietic inhibitors including Exodus (or MIP-3α or LARC) (Hromas *et al* 1997b), Exodus-2 (also known as 6C-Kine or SLC) (Hromas *et al* 1997a), myeloid progenitor inhibitory factor-2 (MPIF-2) (Patel *et al* 1997) and MIP related protein-2 (MRP-2) also known as MPIF-1 or CCF18 (Youn *et al* 1995). With the exception of

MPIF-2, all these reports of actions as putative inhibitory factors have come from the Broxmeyer laboratory. Broxmeyer *et al* (1993) have also previously suggested that in addition to MIP-1α, MCP-1 (and some CXC chemokines) can also function as an inhibitor of haemopoietic progenitor cell proliferation. This observation is in contradiction to a previous report from our laboratory (Graham *et al* 1993), in which only MIP-1α, and MIP-1β to a lesser extent, are active as inhibitors. Exactly why these contradictions exist is uncertain, but they may reflect differences resulting from the different types of assay used by each group. Nevertheless it remains a possibility that other future reports will confirm that many of these novel CC chemokines also function as haemopoietic inhibitors.

3.7 Chemokine Receptors

A great deal of research has been directed at trying to understand how various chemokines interact with their cognate receptors. It is now evident that the chemokine receptors belong to the superfamily of seven transmembrane spanning G protein-coupled receptors (7TMsGPCR) and a multitude of chemokine receptors have now been cloned. Recently, the fields of chemokine biology and virology have surprisingly merged. This has arisen through many outstanding contributions and resulted in the identification of an essential role for chemokines and chemokine receptors in the pathogenesis of HIV infection. A brief overview of the various known chemokines, together with their significance to viral pathogenesis is presented in the following sections.

3.7.1 Early characterisation of receptors

The first chemokine receptors to be identified were those for IL-8, and were named IL-8R_A (Holmes *et al* 1991) and IL-8R_B (Murphy and Tiffany). IL-8 binds to both with high affinity, but IL-8R_A is specific for IL-8, whereas IL-8R_B binds other CXC chemokines (see table 3.2). With the cloning of these receptors it became possible to design degenerate oligonucleotide PCR primers, based on conserved sequences within the IL-8 receptors and other 7TMsGPCRs. Neote *et al* (1993a) and Gao *et al* (1993), subsequently cloned a receptor, CC-CKR1, from U937 cells and PMA differentiated HL60 cells, which bound MIP-1α, MIP-1β, RANTES and MCP-1

Table 3.2 : The Human Chemokine Receptor Family and their Ligands

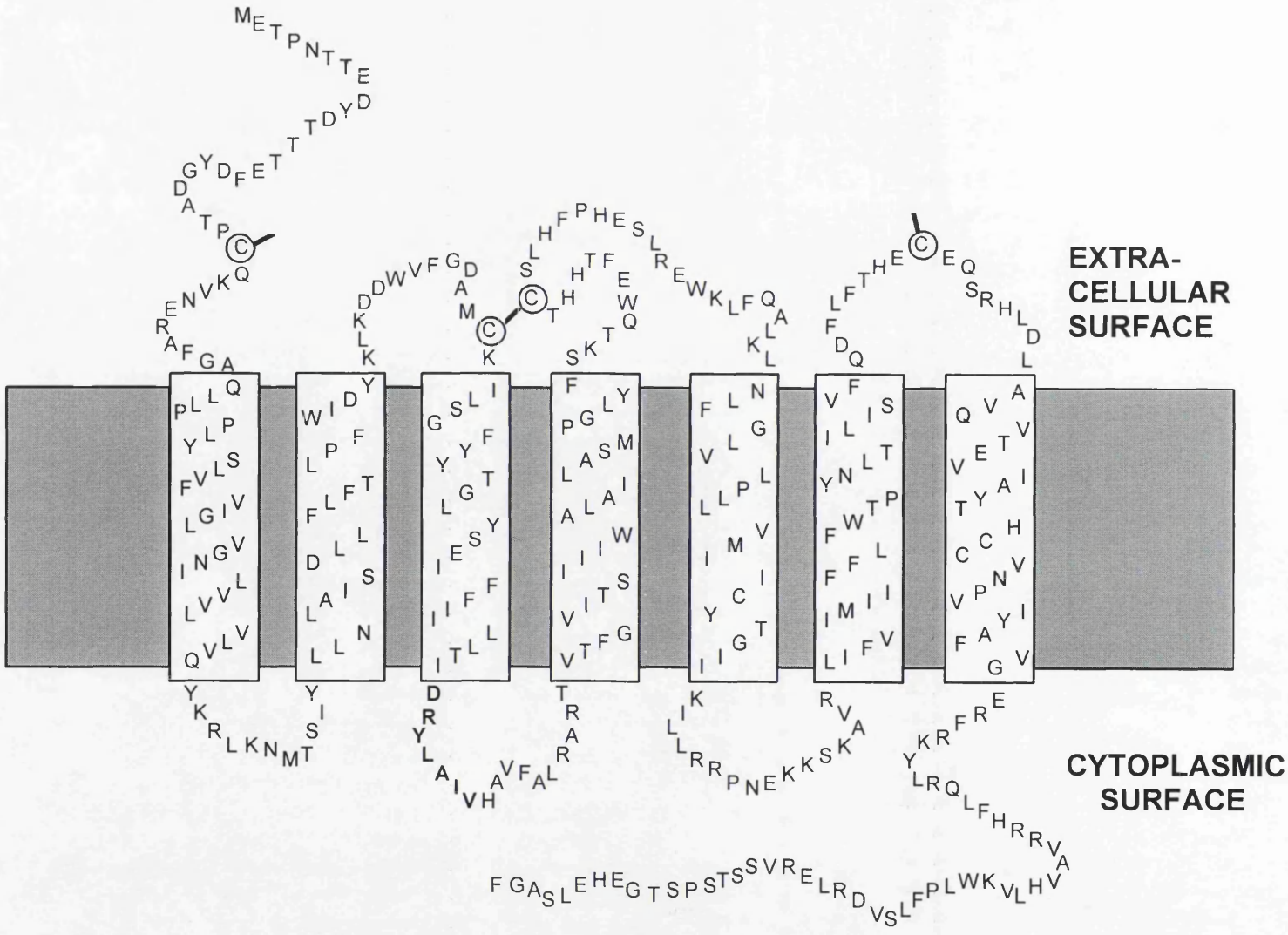
Receptor	Ligands that Bind
CCR1	MIP-1α, RANTES , MIP-1β, MCP-3, MCP-2
CCR2	MCP-1, MCP-2, MCP-3, MCP-5
CCR3	Eotaxin, RANTES, Eotaxin 2, MCP-2 ?, MCP-4 ?
CCR4	TARC, MDC (MIP-1α, RANTES, MIP-1β, MCP-1)
CCR5	RANTES, MIP-1α, MIP-1β
CCR6	MIP-3α
CCR7	MIP-3β
CCR8	I-309
D6	MIP-1α, MIP-1β, MCP-1, 2, 3 and 4, Eotaxin, HCC-1 RANTES
CXCR1	IL-8
CXCR2	IL-8, Gro-α, Gro-β, Gro-γ, NAP-2, ENA-78, GCP-2
CXCR3	IP-10, Mig
CXCR4	SDF-1
CX_3CR1	Fractalkine

with various affinities and this became the first CC chemokine receptor. Many human and murine receptors have been cloned since and the nomenclature was changed in 1996, to accommodate these. Receptors are now prefixed with CXC or CC, depending on whether they bind CC or CXC chemokines. Thus, IL-$8R_A$ and IL-$8R_B$ became CXCR1 and CXCR2 respectively, whilst CC-CKR1 became CCR1. It is now evident that many CC and CXC receptors bind multiple ligands, with varying affinities. To date no CC chemokine has been reported to bind to a CXC receptor and vice versa, with the exception of the Duffy antigen receptor (discussed in section 3.8) and certain virally encoded proteins. The assignation of ligands is therefore complex and made further so by differences between species. To simplify this, the known human CC and CXC chemokine receptors and their known ligands have been summarised in table 3.2.

3.7.2 The 7TMsGPCR superfamily

The chemokine receptors identified to date are all members of the 7TMsGPCR superfamily that also includes receptors for other proinflammatory mediators such as C5a, as well as the best characterised member, the β2-adrenergic receptor. The proposed structure of one such chemokine receptor, CCR1, is shown in figure 3.1. Binding of the cognate ligand results in activation of heterotrimeric G protein complexes, followed by dissociation of the α subunit from the βγ subunit which leads to activation of phospholipase C (PLC) β1 and β2. PLC activation then results in the generation of the second messengers diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). These secondary messengers then trigger a signalling cascade, in which DAG activates protein kinase C, whilst IP3 mobilises calcium from intracellular stores in the cytoplasm and ultimately gives rise to responses such as chemotaxis, degranulation and the respiratory burst. Many different forms of G protein subunit exist and fall into two classes, the G_q class includes $G\alpha_q$, Gα11, Gα14, Gα15 and Gα16 (Simon *et al* 1991) and the G_i class that includes G_i2 and G_i3. The Gq class are all sensitive to pertussis-toxin (PTX), whilst the G_i proteins are PTX-insensitive and appear to directly activate PLCβ2 through the G_i-Gβγ-PLCβ2 pathway via release of Gβγ. In general chemokine receptors are PTX-sensitive, though there are exceptions such as the IL-8 receptors which can function in a PTX-insensitive manner

Figure 3.1: Structure of CCR1: A 7 transmembrane spanning G-protein coupled receptor



(Wu *et al* 1993). Research is currently ongoing to further understand the interaction between chemokines, their receptors and the different G proteins. Recent work by Kuang *et al* (1996), suggests that the CC receptors show different specificities in coupling Gα class proteins (unlike the CXC receptors) and implies that they may have distinct functions *in vivo*, a notion supported by the fact that there is obvious differential expression of Gα proteins.

3.7.3 Receptors for the CC chemokines

3.7.3.1 CCR1

As can be seen from table 3.2 CCR1 is a receptor for multiple chemokines. In general it appears to be a receptor for MIP-1α and RANTES (Neote *et al* 1993a), although MCP-2 and MCP-3 are functional ligands too (Ben-Baruch *et al* 1995). CCR1 appears to be expressed on a wide variety of cell types, including primary monocytes/macrophages and cell lines, neutrophils, B and T lymphocytes, eosinophils and astrocytes (Neote *et al* 1993a, Post *et al* 1995, Tanabe *et al* 1997). A couple of studies have attempted to address the *in vivo* function of CCR1, through generation of CCR1 homozygous null mice. In the first of these, by Gao *et al* (1997), the mice were phenotypically normal. There was evidence however, for disordered steady state, *in vitro* induced trafficking and proliferation of myeloid progenitors in $CCR1^{-/-}$ mice. Furthermore, mature neutrophils from $CCR1^{-/-}$ mice failed to under chemotaxis *in vitro* in response to MIP-1α. These results suggest that CCR1 has a role in haemopoiesis and inflammation *in vivo*. The haemopoietic abnormalities are surprising in the light of the normal haemopoiesis shown by MIP-$1\alpha^{-/-}$ mice (Cook *et al* 1995) and studies from our laboratory that suggest CCR1 is not the receptor through which MIP-1α inhibits stem cell proliferation (Graham *et al* 1996). One reason for these apparent discrepancies is that the study by Gao *et al* used an *in vitro* stimulus (LPS) to assess the trafficking and proliferation of myeloid progenitor cells. Therefore this does not prove that CCR1 is critical for *in vivo* control of myeloid progenitor cell trafficking and proliferation. In another study, performed by Gerard and collegues using CCR1 deficient mice, CCR1 was shown to be important for progression of inflammation (Gerard *et al* 1997). The authors reported that deletion of CCR1 was associated with

protection from Pancreatitis-associated lung injury and moreover that this was associated with decreased levels of TNF-α.

3.7.3.2 CCR2

The CCR2 receptor was first cloned by Charo *et al* (1994) from the MonoMac6 cell line. The receptor appears to exist in two forms, CCR2A and CCR2B, generated by alternative splicing of a single gene. They appear to differ only in their C-terminal tails, although CCR2A is not as well expressed on the cell surface. CCR2B appears to be an MCP-specific receptor and does not bind either MIP-1α, MIP-1β, or RANTES (Myers *et al* 1995). Through the use of truncation mutants and site-directed mutagenesis, the Charo group has been very productive in determining regions involved in interactions between CCR2 and in particular, MCP-1 (Monteclaro and Charo 1996, Monteclaro and Charo 1997, Wong *et al* 1997, Arai *et al* 1997). Most recently, a non-redundant role for CCR2 in macrophage recruitment and host defence against bacterial pathogens was reported (Kurihara *et al* 1997, Kuziel *et al* 1997)

3.7.3.3 CCR3

CCR3 is a receptor with a more restricted expression pattern. It appears highly expressed on eosinophils (Kitaura *et al* 1996) and basophils (Uguccioni *et al* 1997). Limited expression has also been reported on elicited macrophages and neutrophils (Gao *et al* 1996), although the functional relevance of this is questionable. Human CCR3 is very specific in binding only eotaxin, MCP-3, MCP-4, and RANTES, all of which are eosinophil chemoattractants. This further reinforces the notion of CCR3 being an eosinophil/basophil specific receptor. However, a very recent study suggests a wider role in the allergic response, in that CCR3 appears to be expressed on T_h2 cells and moreover, that eotaxin was able to stimulate increases in intracellular calcium and chemotaxis of $CCR3^+$ T cells (Sallusto *et al* 1997). This finding is particularly interesting, because it suggests that eotaxin can attract eosinophils, basophils and T_h2 cells, three of the most important cells for development of an inflammatory allergic response.

3.7.3.4 CCR4

Human CCR4 was originally cloned from a human immature basophilic cell line (Power *et al* 1995) and was found to be expressed strongly in the thymus, as well as in T and B lymphocytes, peripheral blood monocytes and in IL-5 stimulated basophils. Originally, human CCR4 was reported to be a functional receptor for MIP-1α, RANTES and MCP-1, however it is now uncertain whether this is indeed true. The murine homologue was subsequently cloned (Hoogewerf *et al* 1996) and shown to have similar binding characteristics and tissue expression as its human homologue. Imai and collegues have demonstrated that the T cell directed CC chemokine TARC, was a specific ligand for CCR4 and did not bind to any other known receptors (Imai *et al* (1997). More recently this group reported that macrophage-derived chemokine (MDC) was also a functional ligand for CCR4 Imai *et al* (1998).

3.7.3.5 CCR5

CCR5 was first cloned by Samson *et al* (1996a) and subsequently by Raport *et al* (1996) and Combadiere *et al* (1996). CCR5 is abundantly expressed on macrophages and T cells and appears to be a relatively specific receptor, binding only MIP-1α, MIP-1β and RANTES (Raport *et al* 1996). Recently, Samson *et al* (1997) demonstrated that a region within the second extracellular loop of CCR5 was critical for high affinity binding of these ligands and subsequent functional response. CCR5 is now the focus of intense study, as a result of its recent identification as a co-factor for HIV entry and this is discussed further in section 3.9.

3.7.3.6 Other CC Receptors

CCRs 1-5 are the most studied CC chemokine receptors to date, but several other receptors have recently been identified. CCR6 appears to show relatively restricted expression in B and T lymphocytes and was strongly expressed in $CD34^{+}$-derived dendritic cells, but not on monocyte-derived ones, nor on monocytes, granulocytes or NK cells (Baba *et al* 1997, Greaves *et al* 1997). CCR7 was recently reported as a receptor for the novel chemokine MIP-3β or ELC (Yoshida *et al* 1997) and is strongly expressed in B and T lymphocytes (Birkenbach *et al* 1993). The receptor for I-309, a human CC chemokine was recently identified and named CCR8 (Tiffany *et al* 1997, Stuber Roos *et al* 1997). D6 is a recently characterised receptor,

cloned in our laboratory (Nibbs *et al* 1997). It is unusual in having an altered DRYLAIV motif in the second intracellular loop, which is normally highly conserved and which appears critical for signalling. Murine D6 is the highest affinity receptor for MIP-1α identified to date. The human homologue of this was recently cloned (Nibbs *et al* 1998) and is also a high affinity receptor for MIP-1α, and also binds MIP-1β, MCPs 1-4, eotaxin and HCC-1. Unusually however, D6 does not appear to flux calcium in HEK293 cells. The functional significance of this and the other unusual characteristics of D6 are unclear at present.

3.7.4 Receptors for the CXC Chemokines

As indicated in section 3.7.1, CXCR1 is a receptor specific for IL-8, whilst CXCR2 is also a receptor for IL-8, as well as for other CXC chemokines. Recently, expression of CXCR2 was demonstrated on subsets of neurons within the CNS (Horuk *et al* 1997). Loetscher *et al* (1996) cloned the receptor (CXCR3) for IP-10 and Mig, that appears to be mainly expressed in activated T cells and which does not bind to any of the other known CXC chemokines. Murine CXCR4, cloned by Nagasawa *et al* (1996b) was shown to be the receptor for SDF-1 and the murine homologue of the human fusin HIV entry cofactor identified by Feng *et al* (1996). The role of CXCR4 in HIV entry will be discussed in section 3.9. CXCR4, like CCR2, appears to be expressed in two forms (CXCR4A and B) derived from a single gene by alternative splicing (Heesen *et al* 1996).

3.8 The Duffy Antigen for Chemokines (DARC)

One of the most unusual identified chemokine receptors is DARC. The Duffy antigen is an erythrocyte cell surface receptor for the malarial parasite, *plasmodium vivax* (reviewed by Hadley and Peiper 1997). Surprisingly, a promiscuous chemokine receptor that bound both CXC and CC chemokines (with the exception of MIP-1α) was also identified on erythrocytes (Neote *et al* 1993b) and subsequently shown to be identical to the Duffy antigen (Horuk *et al* 1993). DARC as it was named, is also expressed on the endothelial cell lining some postcapillary venules and on Purkinje cells in the cerebellum of the brain (Horuk *et al* 1996). The precise function of DARC *in vivo* is still unclear, as it does not appear to present chemokines in an active form to receptors, nor does it appear to signal. It may instead function to soak up excess

chemokines from the circulation. Its strong conservation between species and the observation that Duffy blood group negative individuals (who are malaria resistant) do still express Duffy on endothelial cells of postcapillary venules, suggests that it does serve a critical role *in vivo*. Very recently, it was reported by Rot and his colleagues, that IL-8 is actually internalised by venular endothelial cells (EC) and transcytosed to the luminal surface where it is presented to adherent leukocytes on the membrane of EC (Middleton *et al* 1997). It is possible that DARC plays a role in this chemokine transcytosis and presentation.

3.9 Chemokines, their Receptors and Significance to HIV Pathogenesis

Beginning in late 1995, the fields of chemokine and AIDS research have rapidly merged, in what has already become one of the most significant breakthroughs in AIDS research over the last 15 years. The review by D'Souza and Harden (1996) provides an excellent summary of this. The following section briefly overviews some of the reports that led to the discovery of the critical role of chemokines and their receptors in HIV pathogenesis.

One of the great elusive mysteries of AIDS research has been the missing cofactor / receptor required for entry of HIV-1 into cells in addition to the previously identified CD4 molecule (Dalgleish *et al* 1984). Cocchi *et al* (1995) demonstrated that MIP-1α, MIP-1β, RANTES were three soluble factors released by $CD8^+$ T cells, that suppressed the entry of primary strains of HIV-1 and provided the first clues to solving this puzzle. In 1996, Feng *et al* made a seminal discovery and identified the second receptor necessary for HIV-1 entry into T cell lines, so called T-tropic viruses. They named this protein fusin, because it aided HIV fusion (Feng *et al* 1996). Fusin is identical to CXCR4, the receptor for SDF-1 (Bleul *et al* 1996b), and was only permissive for entry of T-tropic strains and not for macrophage-tropic (M-tropic) viruses. Subsequently, five groups simultaneously reported that the cofactor for M-tropic viruses was the CCR5 receptor and that CCR2b and CCR3 also functioned as cofactors, though less effectively (Dragic *et al* 1996, Deng *et al* 1996, Alkhatib *et al* 1996, Doranz *et al* 1996, Choe *et al* 1996). It is now clear that during infection some viruses evolve to become dual-tropic and can utilise both CC and CXC receptors (Doranz et al 1996), whilst some strains of HIV-2 can use CXCR4 in a CD4 independent fashion (Endres *et al* 1996). Intense study is now directed at determining

critical interactions between different regions of CD4, HIV and various receptors. It now appears that the gp120 protein of HIV induces a complex between CD4, gp120 and CXCR4 (Lapham *et al* 1996). Others have shown similar interactions for CCR5, in which gp120 interacts directly with CCR5, whilst CD4 appears to promote these interactions (Wu *et al* 1996). The V3 domain of HIV gp120 appears critical for this interaction (Cocchi *et al* 1996).

It has been demonstrated that receptor signalling and internalisation are not required for CCR5 to function as an HIV-1 entry co-factor (Gosling *et al* 1997, Farzan *et al* 1997a, Aramori *et al* 1997). Interestingly, Weissman *et al* (1997) have suggested that recombinant M-tropic HIV and SIV envelope proteins can induce a signal through CCR5 on $CD4^+$ T cells, which mediates T cell chemotaxis. Davis *et al* (1997) recently reported that chemokines and HIV-1 envelope glycoproteins induced tyrosine phosphorylation of the protein tyrosine kinase, Pyk2 (Davis *et al* 1997). This response required CXCR4 and CCR5 to be accessible at the cell surface. The significance of these intracellular signalling events are unclear at present.

Several reports have established a genetic correlation of protection. Paxton *et al* (1996) reported that the $CD8^+$ T cells of individuals who remained uninfected, despite multiple high-risk exposures, had greater antiviral activity than those of unexposed controls. Their $CD4^+$ T Cells also appeared less susceptible to M-tropic viral infection. This protection appeared to relate to the activity of the suppressive factors MIP-1α, MIP-1β, and RANTES. Several groups subsequently demonstrated a genetic basis for protection of multiply-exposed high-risk individuals against HIV-1 infection. Some exposed, uninfected individuals were shown to have a homozygous defect in their CCR5 gene. This was the result of a 32 bp deletion in the CCR5 allele, resulting in expression of a truncated CCR5 protein, that is not expressed on the cell surface (Samson *et al* 1996b, Liu *et al* 1996). A heterozygous allelic defect may or may not provide a limited protective effect (Dean *et al* 1996, Huang *et al* 1996). It is clear however, that the protective role of the CCR5 deletion allele is not absolute. Although rare, individuals who are homozygous for this defect can be infected (Biti *et al* 1997), perhaps if the original infecting virus evolves to utilise CXCR4 (Michael *et al* 1997a). Reports have also identified allelic variants of CCR2, though the existence of any protective role is unclear (Michael *et al* 1997b, Smith *et al* 1997).

As well as genetic variants in cofactor receptors, the role of endogenous suppressive chemokines in protection against HIV is crucial. Unfortunately determination of their roles is not so simple, whilst it is clear that SDF-1 can suppress T-tropic infection via CXCR4 (Bleul *et al* 1996b) and the CC chemokines MIP-1α, MIP-1β and RANTES suppress M-tropic infection (Cocchi *et al* 1996), it is unlikely that these are the only suppressive chemokines (Moriuchi *et al* 1997). Indeed Pal *et al* (1997) recently identified another CC chemokine, macrophage derived chemokine (MDC), as being suppressive. Most recently a rare genetic variant of SDF-1 was identified (Winkler *et al* 1998). This polymorphism (SDF-1 3'A) was in the 3' untranslated region of SDF-1 and the homozygous variant provided a protective effect against the onset of AIDS, whilst the recessive variant was also protective, particularly during the later stages of disease progression. This protective effect was even more effective than those conferred by the CCR5 (CCR5 del32) and CCR2 (CCR2-641) receptor variants discussed previously in this section. It was speculated that this protective effect may be due to an enhanced level of SDF-1 production, though this remains to be proven.

Although CCR5 and CXCR4 appear to be the primary cofactors along with CCR2b, and CCR3 in the CNS (He *et al* 1997), other HIV entry co-receptors will no doubt be identified. Indeed, several orphan receptors have already been identified (Farzan *et al* 1997b), along with an unusual chemokine-like receptor, STRL33 that appears to function as a fusion-cofactor for both M and T-tropic HIV-1 (Liao *et al* 1997).

Clearly the field of HIV-related chemokine biology is in its infancy, but already chemokine antagonists and other strategies are being developed in attempts to produce therapeutic solutions for intervention and perhaps ultimately a cure for HIV infection.

3.10 Virally Encoded Chemokine and Chemokine Receptor Homologues

As a result of the recent interest in chemokines and viral pathogenesis, it has now emerged that many viruses encode homologues of chemokine and chemokine receptor-like genes. The first evidence for this was reported in 1994, with the identification of a chemokine receptor-like gene in the US28 open reading frame of the human cytomegalovirus (CMV). The US28 gene encodes a receptor that was

functional in response to MIP-1α, RANTES and MCP-1 (Gao and Murphy 1994). A viral homologue of the CXCR2 receptor is encoded by the ECRF3 gene of herpesvirus saimiri (Ahuja and Murphy 1993).

More recently, human herpesvirus 8 (HHV8) or Kaposi's sarcoma-associated herpesvirus (KSHV) has been shown to encode several viral homologues. vMIP-I and vMIP-II have homology to MIP-1α, whilst the virus also encodes a viral form of IL-6, vIL-6 (Moore *et al* 1996). Arvanitakis *et al* (1997) have recently identified a KSHV encoded G protein-coupled receptor-like gene, with homology to the IL-8 receptors and ECRF3 gene. This receptor-like protein appears to bind both CC and CXC chemokines. Unusually, this homologue appears to be constitutively active and stimulated cell proliferation, suggesting it could be a potential viral oncogene. This oncogenic potential was recently confirmed, by Bais *et al* (1998), who demonstrated that signalling via this G-protein-coupled KSHV encoded receptor resulted in cell transformation, tumourigenicity and a switch to an angiogenic phenotype mediated by vascular endothelial growth factor (VEGF). vMIP-II was recently shown to function as a broad spectrum antagonist and inhibited HIV-1 entry via CCR3, CCR5 and CXCR4 (Kledal *et al* 1997), as well as being a chemoattractant for $CCR3^+$ eosinophils (Bosshoff *et al* 1997).

Molluscum contagiosum virus (MSV) types I and II were recently shown to encode a chemokine-like gene. The products were able to block the chemotactic responses of monocytes to MIP-1α, but were not chemotactic themselves. These proteins were also active as inhibitors of human progenitor cell proliferation, with even greater potency than human MIP-1α (Krathwohl *et al* 1997).

Lalani *et al* (1997) purified a gamma interferon receptor homologue, M-T7, encoded by myxoma virus and demonstrated that it interacted with the heparin binding domains of both CC and CXC chemokines. M-T7 has no homology with any known chemokine receptors and the significance of it's apparent abilities are unclear at present.

Clearly many viruses have evolved or acquired chemokine-like and chemokine receptor-like genes. In general, viruses do not have the spare coding capacity to encode such genes without good reason, but at present the role that these play in viral pathogenesis is unknown. Further study of the virally encoded homologues may well

enable further understanding of the role of chemokines and their receptors in the immuno-haemopoietic system.

3.11 In Summary

It should be evident by now, that the control of the immuno-haemopoietic system is enormously complex. Central to its regulation, are a multitude of growth factors and cytokines, together with their cognate receptors. These form an interacting network, that ultimately functions at every level from early development, through to the control of constitutive and inducible haemopoiesis. Within this network, the chemokine MIP-1α, is a particularly important mediator. It functions as a regulator of both stem cell proliferation and inflammation. Recent studies have highlighted the importance of MIP-1α and other chemokines to viral pathogenesis and enabled great advances in our understanding of MIP-1α, particularly concerning interactions with its various receptors. However, little is known regarding the mechanisms that operate to regulate the expression of MIP-1α and its receptors. This is particularly true in the context of haemopoiesis and the interaction of MIP-1α with other growth factors within the bone marrow microenvironment. This therefore presents an exciting area for further research.

AIMS OF THESIS

In order to fully understand the complex process of haemopoiesis, it is necessary to understand the role that haemopoietic growth factors and cytokines play in regulating this process. It is now evident that a large interacting network of such regulatory cytokines exists and these have been implicated in various aspects of haemopoietic control, within the haemopoietic stem cell compartment. It is also apparent that both stimulatory and inhibitory molecules function within this network. Our group has a particular interest in one such inhibitor of haemopoietic stem cell proliferation, namely macrophage inflammatory protein-1 alpha (MIP-1α). Current evidence suggests that MIP-1α is member of this network and therefore in order to fully understand the role of MIP-1α in haemopoietic stem cell inhibition, it is also necessary to further understand how its expression within the bone marrow is regulated. Recently our laboratory identified an endogenous, reciprocal relationship in bone marrow-derived macrophages between MIP-1α and transforming growth factor-beta (TGF-β), in which it appears that TGF-β acts to suppress MIP-1α expression. Whilst the level of MIP-1α expression within the bone marrow is very low, it is detectable and this implies that stimulatory mechanisms may exist to overcome this TGF-β mediated suppressive block. The aim of this study therefore, was to investigate the potential effects of haemopoietic growth factors on the regulation of MIP-1α expression. It was believed probable that within the bone marrow microenvironment, positively acting factors were also functional in the regulatory control of MIP-1α expression. Investigation of the effects of such positively acting factors and any relationship between them, MIP-1α and TGF-β, might enable further understanding of the circumstances under which MIP-1α expression might be altered, and thus provide further insight into its function within the haemopoietic stem cell or inflammatory cell compartments.

PART II: MATERIALS AND METHODS

CHAPTER 4: MATERIALS

Table 4.1: Plasmids for Generation of Probe Fragments

Plasmid name	Identity of Insert	Insert size	Excision sites	Source	Address
pSK.β-Actin	Murine β-Actin cDNA	1200 bp	Pst I	D. Jarmin	Beatson Institute
pSK.LD78	Human MIP-1α cDNA	~300 bp	Pst I	S. Wylie	Beatson Institute
pMTX-MIP-1α	Murine MIP-1α cDNA	~300 bp	EcoR I / Xho I	S. Wylie	Beatson Institute
pMTX-MIP-1β	Murine MIP-1β cDNA	~300 bp	EcoR I / Xho I	S. Wylie	Beatson Institute
pSK.mCCR1	Full murine CCR1 cDNA	1346 bp	BamH I / Not I	Dr. R. Nibbs	Beatson Institute
pSK.mCCR3	Full murine CCR3 cDNA	1392 bp	BamH I / Not I	Dr. R. Nibbs	Beatson Institute
pSK.mCCR4	Full murine CCR4 cDNA	1364 bp	BamH I / Not I	Dr. R. Nibbs	Beatson Institute
pSK.mCCR5	Full murine CCR5 cDNA	1095 bp	BamH I / Not I	Dr. R. Nibbs	Beatson Institute
pSK.mD6	Full murine D6 cDNA	1621 bp	BamH I / Not I	Dr. R. Nibbs	Beatson Institute
pMARC	Full murine MCP-3 (MARC) cDNA	776 bp	BamH I / Xho I	Dr. T. Baumruker	Novartis F.I. (Vienna, Austria)
pcJE-1	Full JE cDNA	578 bp	EcoR I	Dr. B. Rollins	(Dana-Farber Cancer Institute MA, USA)
pcDNA-3 hCCR1	Full human CCR1 cDNA	1065 bp	Hind III / Not I	Dr. T. Schall	MMRI (Palo-Alto, CA, USA)

Table 4.2: Plasmids for Use in RT-PCR Analysis

Plasmid name	Identity of Insert	Source	Address
pCDM8.mIL-5Rα	Full murine IL-5R-α chain cDNA	Dr. G. Plaetnick	Roche Research (Gent, Belgium)
pCRII.rGAPDH	Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (containing sequences between bp 300 - 1020)	M. Walker	Beatson Institute
pSK.mCCR1 pSK.mCCR3 pSK.mCCR4 pSK.mCCR5 pSK.mD6	Details as per Table 4.1		

Table 4.3: PCR Primers- Identity

Name	Orientation	Amplifies	Comments
DJ03	5'	Murine IL-5Rα	Amplifies both membrane bound and
DJ04	3'		soluble IL-5Rα chain fragments
DJ09	5'	Murine MIP-1α	Crosses 1st Intron/exon boundary
DJ10	3'		(247 bp mRNA, ~1 kb genomic)
DJ11	5'	Murine GAPDH	
DJ12	3'		
DJ17	5'	Human MIP-1α	
DJ18	3'		
DJ23	5'	Murine D6	
DJ24	3'		
DJ25	5'	Murine CCR1	
DJ26	3'		
DJ27	5'	Murine CCR3	
DJ28	3'		
DJ29	5'	Murine CCR4	
DJ30	3'		
DJ31	5'	Murine CCR5	
DJ32	3'		
DJ33	5'	Murine CCR2	
DJ34	3'		
RNPCR.βA5	5'	Murine and human	Mouse mRNA= 247 bp
RNPCR.βA3	3'	β-Actin	

Table 4.4: PCR Primers- Sequences

DJ03	5'	GCCATTGACCAAGTGAATCC	3'
DJ04	5'	GTGGAATTTCCCATGACTTC	3'
DJ09	5'	ATGAAGGTCTCCACCACTGCC	3'
DJ10	5'	TCAGGCATTCAGTTCCAGGTC`	3'
DJ11	5'	TGAGTATGTCGTGGAGTCTAC	3'
DJ12	5'	GGCCATGTAGGCCATGAGGTC	3'
DJ17	5'	CACTTGCTGCTGACACG	3'
DJ18	5'	CAACCAGTCCATAGAAGAGG	3'
DJ23	5'	GTTTTCTTCATGCTGTGGTTCC	3'
DJ24	5'	GGAAGAGACAGTAATGAGTAAGGC	3'
DJ25	5'	CTTCTATTCTTCCTCCTCTGGAC	3'
DJ26	5'	GAGCTCATGTTCTCCTGTGGA	3'
DJ27	5'	CTTGCAGGACTGGCAGCATTG3'	
DJ28	5'	CAAAAATAAGACGGATGGCCTTG	3'
DJ29	5'	GAATGAGAAGAAGAACAGAG	3'
DJ30	5'	CTGGACATGTCAGCCGAGTAGAC	3'
DJ31	5'	CTTTACCAGATCTCAGAAAGAAGG	3'
DJ32	5'	GAGTCTCTGTTCCTGCATGG	3'
DJ33	5'	CAAAGGAAATGGAAGACAATA	3'
DJ34	5'	GAAATGTTGGGTTGGCTC	3'
RNPCR.βA5	5'	TCCATCATGAAGTGTGACGT	3'
RNPCR.βA3	5'	TACTCCTGCTTGCTGATCCAC	3'

4.5 Tissue Culture Supplies

Supplier	Address	
Beatson Institute Central Services	(Glasgow, UK)	Sterile phosphate buffered saline (PBS) Sterile glassware and pipettes
R2, Beatson Institute	(Glasgow, UK)	L929 CM
Becton Dickinson, UK Ltd.	(Plymouth, UK)	Falcon tubes (15 ml and 50 ml)
Costar	(Cambridge, MA USA)	6 and 24 well culture plates Cell scrapers
Gelman Sciences	(Northampton, UK)	Sterile acrodisc syringe filters (0.2 μm and 0.45 μm)
Gibco Life Technologies	(Paisley, UK)	MEM alpha stock L-glutamine (200 mM) Sodium pyruvate (100 mM) Sodium bicarbonate (7.5 %) MEM Non essential amino acids (100x) Hepes (1 M) Trypsin (2.5 %)
Greiner Labortechnik	(Stonehouse, UK)	Endotoxin-free plugged pipetteman tips
Nalge Nunc International	(Roskilde, Denmark)	Tissue Culture Flasks Cryotubes
Sigma Chemical Co.	(Poole, UK)	Endotoxin-free Dulbecco's PBS (10 x) 2-Mercaptoethanol (2mM stock in PBS) Donor Horse Serum Bovine Serum Albumin, FractionV, 7.5% RPMI-1640 Medium Dulbecco's MEM
TCS Biologicals Ltd.	(Buckingham, UK)	Foetal Calf Serum

4.6 Cytokines and Antibodies

The murine MIP-1α PM2 deletion mutant was the form of MIP-1α used in these studies and was as described in Graham *et al* (1994).

Supplier	Address	
R&D Systems Europe	(Abingdon, UK)	Recombinant anti-murine MIP-1α antibody
		Recombinant human TGF-β1
		Recombinant murine GM-CSF
		Recombinant human GM-CSF
		Recombinant murine IL-3
		Recombinant murine IL-5
		Recombinant murine G-CSF
		Recombinant murine IL-6
Sigma Immunochemicals	(Poole, UK)	Anti-goat IgG (HRP-conjugated)
Dr. N. Hole	(University of Durham, UK)	COS-cell derived murine LIF

4.7 Kits

Supplier	Address	
Amersham International	(Amersham, UK)	ECL Western blotting detection kit
Invitrogen BV	(Leek, The Netherlands)	Micro-FastTrack mRNA isolation kit
Perkin Elmer	(Foster City, CA, USA)	GeneAmp® RNA PCR core kit
Pharmacia Biotech	(St Albans, UK)	Ready-To-Go DNA labelling kit
Qiagen Inc.	(Chatsworth, CA, USA)	QIAGEN plasmid preparation kits
		QIAquick gel extraction kit

4.8 Membranes, Paper and X-ray Film

Supplier	Address	
Amersham International	(Amersham, UK)	Hybond N nylon membranes
Genetics Research, Ltd	(Felstead Dumnow, UK)	Saranwrap
Millipore Corp.	(Bedford, USA)	Immobilon-P transfer membranes
Vernon-Carus, Ltd.	(Preston, UK)	Gauze swabs
Whatman International Ltd.	(Maidstone, UK)	3MM blotting paper
Kodak Scientific Imaging Sytems Ltd.	(Cambridge, UK)	X-OMAT AR X-ray film
Technical Photo Systems	(Cumbernauld, UK)	Fuji RX Medical X-ray film

4.9 Nucleotides

Supplier	Address	
Amersham International	(Amersham, UK)	[α-^{32}P]-dCTP: 3000 Ci/mmol
Dupont NEN	(Stevenage, UK)	^{125}Iodine: 100 mCi/ml

4.10 Protein and DNA Markers

Supplier	Address	
Amersham International	(Amersham, UK)	Rainbow coloured protein molecular weight markers: (High m.w. : 14,300-220,000 Da) (Low m.w. : 2,300-46,000 Da)
Gibco BRL	(Paisley, UK)	RNA ladder (0.24-9.5 kb)

		Low DNA mass ladder
		123 bp DNA ladder
		1 Kb DNA ladder

4.11 Enzymes, Enzyme Buffers and Enzyme Inhibitors

Supplier	Address	
Gibco BRL	(Paisley, UK)	All Restriction enzymes and buffers (unless stated otherwise)
		T4 DNA ligase (1 U/µl)
		5x ligation buffer
Boehringer Mannheim	(Mannheim, Germany)	RNase free DNase I (10 U/µl)
Invitrogen BV	(Leek, The Netherlands)	RNaseZap (RNase inhibitor)
Pharmacia Biotech	(St Albans, UK)	One-phor-all plus™ restriction enzyme buffer

4.12 Columns

Supplier	Address	
Pharmacia Biotech	(St Albans, UK)	NICK columns

4.13 Chemicals

Supplier	Address	
Boehringer Mannheim	(Mannheim, Germany)	MOPS
DIFCO Laboratories	(Michigan, USA)	Bactoagar
Fisons Scientific Equipment	(Loughborough, UK)	Formaldehyde (38 % w/v)
		Glycerol

GIBCO BRL	(Paisley, UK)	Agarose TRIzol reagent Phenol/Chloroform/Isoamyl alcohol (25:24:1, v/v) (pH 8.0)
James Burroughs Ltd.	(Witham, UK)	Ethanol
Pierce and Warriner	(Chester, UK)	Iodogen reagent
Severn Biotech Ltd.	(Kidderminster, UK)	Design-a-gel 30 % (w/v) Acrylamide, 0.8 % (w/v) bis-Acrylamide solution
Sigma chemical Co.	(Poole, UK)	Ampicillin Bromophenol blue Diethylpyrocarbonate (DEPC) Dithriothreitol (DTT) Ethidium bromide DNA samples (10mg/ml) RNA samples (1mg/ml, in DEPC treated water) Fura-2-AM TEMED Tween 20
Thornton and Ross	(Huddersfield, UK)	Liquid Paraffin
Fisher Scientific UK Ltd.	(Loughborough, UK)	All other chemicals not listed above

4.14 Bacterial Culture

4.14.1 Solutions

Supplier	Address	
Beatson Institute Central Services	(Glasgow, UK)	Luria-Broth

4.14.2 Host strains

Supplier	Address	
GIBCO BRL	(Paisley, UK)	DH5α competent cells

4.15 Solutions

DEPC-water	0.1 % DEPC in sterile distilled water, shaken and left overnight at room temperature. Autoclaved and allowed to cool.

4.15.1 Agarose gel electrophoresis

1x TE (pH 8.0)	10 mM Tris.HCl (pH 8.0) 1 mM EDTA
50x TAE	2 M Tris-acetate 5 mM EDTA (pH 8.0)
DNA sample loading buffer	50 % Glycerol (v/v) 1x TAE Bromophenol blue to colour

4.15.2 DNase I digestion

DNase I restriction buffer (10x)	50 mM Sodium acetate (pH 5.2) 1 M Magnesium chloride made up in DEPC-water

4.15.3 Northern blotting and hybridisation

10x MOPS buffer (pH 7.0)	0.4 M MOPS (pH 7.0) 0.1 M Sodium acetate 10 mM EDTA
RNA sample loading buffer	50 % Glycerol (v/v) 1x MOPS

	Bromophenol blue to colour made up in DEPC-water
20x SSC	3 M Sodium chloride 0.3 M Sodium citrate
(Pre-)hybridisation buffer	0.25 M Sodium phosphate (pH 7.0) 7 % SDS (w/v)

4.15.3 SDS-Polyacrylamide Gel Electrophoresis (SDS/PAGE) and Western blotting

17.5 % Polyacrylamide gel (resolving)	17.5 % Acrylamide mix (w/v) 375 mM Tris.HCl (pH 8.8) 0.1 % SDS (v/v) 0.1 % Ammonium persulphate (w/v) 0.04 % TEMED (v/v)
5 % Polyacrylamide gel (stacking)	5 % Acrylamide mix (w/v) 125 mM Tris.HCl (pH 6.8) 0.1 % SDS (w/v) 0.1 % Ammonium persulphate (w/v) 0.01 % TEMED (v/v)
Protein sample loading buffer	5 µl 10 % SDS / DTT (sample buffer) 7 µl 50 % glycerol (with Bromophenol blue to colour)
SDS / PAGE running buffer	200 mM Glycine 25 mM Tris-acetate 0.1 % SDS (w/v)
Transfer buffer	40 mM Glycine 50 mM Tris.HCl 0.04 % SDS (w/v) 20 % Methanol (v/v)

4.15.4 Binding studies

Binding buffer	1x MEM alpha medium: [20 % MEM alpha stock (v/v), 1 mM L-Glutamine, 0.1 % Sodium bicarbonate (v/v), 25 % DHS (v/v), 20 % L-929 CM (v/v)]. 25mM HEPES (pH 7.4) 0.2 % Sodium azide (w/v)

4.15.5 Calcium mobilisation studies

HACM buffer	125 mM Sodium chloride 5 mM Potassium chloride 0.025 % BSA, fraction V (w/v) 1 mM Magnesium chloride 20 mM HEPES (pH 7.4) 0.5 mM D-Glucose

CHAPTER 5: METHODS

5.1 Mice

Female B6D2F1 mice, aged between 4-6 weeks at arrival, were used for all experimental procedures. Mice were purchased from Harlan UK, Ltd. (Oxon, UK) and housed within the animal facility at the Beatson Institute.

5.2 Cell Lines

Cell lines were obtained from the sources indicated in table 5.1. All cell lines were maintained at 37°C in a dry atmosphere of 5% CO_2 in air.

5.2.1 THP-1

The human pro-monocytic cell line THP-1, derived from a patient with acute monocytic leukaemia (Tsuchiya *et al* 1980), was maintained in RPMI 1640 + 2 mM glutamine / 0.02 mM β-mercaptoethanol / 10 % FCS in Nunc 75 cm^2 tissue culture flasks. THP-1 cells grow in suspension and were subcultured every 2-3 days, by resuspension in fresh medium at a concentration of 2-9 x 10^5 cells/ml.

5.2.2 BCL1 (clone 13.20-3B3)

The murine chronic B-cell leukaemia cell line BCL1 clone 13.20-3B3, derived from the BCL1 tumour cell line (Gronowicz *et al* 1980) was maintained in RPMI 1640 + 10 mM HEPES / 2 mM glutamine / 0.1 mM non-essential amino acids / 1 mM Sodium pyruvate / 0.05 mM β-mercaptoethanol / 10 % FCS in Nunc 75 cm^2 tissue culture flasks. BCL1 cells are a loosely adherent cell line and were subcultured upon reaching 75-90 % confluence (approximately every 2-3 days), by sharply tapping the flask against the palm of the hand 3-4 times. The detached cells were then resuspended in fresh medium at a subcultivation ratio of 1:3 to 1:5.

5.2.3 HEK 293-hCCR5 stable transfectants

Human embryonic kidney (HEK) 293 cells stably transfected with human CCR5 were maintained in DMEM + 4 mM glutamine / 10 % FCS in Nunc 175 cm^2 tissue culture flasks. Cells were subcultured every 2-3 days, upon reaching 75-90 %

Table 5.1: Tissue Culture Cell Lines

Supplier	Address	Cell line	Type of Cell line
European Collection of Animal Cell cultures (ECACC)	(Salisbury, UK)	THP-1	Human Monocytic leukaemia
American Type Culture Collection (ATCC)	(Rockville, MD, USA)	BCL1 (clone 13.20-3B3)	Murine B-cell leukaemia
Dr. R. J. Nibbs	(Beatson Institute)	HEK hCCR5	Human Embryonic kidney cells, stably transfected with hCCR5

Table 5.2: Analysis of Bone Marrow-Derived Macrophage Population

Day	Percentage of non-specific Esterase Positive Cells
4	82 %
5	96 %
6	98.5 %
7	99 %
8	100 %

confluence, by detaching with trypsin and resuspending in fresh medium at a concentration of 5×10^5 to 1×10^6 cells/ml.

5.3 Bone Marrow-Derived Macrophages

5.3.1 Harvesting of bone marrow

4-6 wk old female B6D2F1 mice were used throughout for the harvesting of bone marrow. Mice were killed by cervical dislocation. The femora were excised using surgical scissors and excess muscle removed from the bone shaft and ends using a tissue soaked in ethanol. Both ends of the femora were then cut off with scissors and the bone marrow cells expelled by flushing through a 21 gauge needle with 2.5 ml per femur of α-modified MEM supplemented with 25 % DHS. Approximately 6-9 x 10^6 bone marrow cells were routinely obtained from each femur using this method.

5.3.2 Derivation of bone marrow-derived macrophages

Bone marrow cells were obtained (as above) and resuspended at 5 x10^4 /ml in α-modified MEM supplemented with 25 % DHS and 20 % L929 CM as source of M-CSF. Following incubation for 7 days at 37°C in a dry atmosphere of 5 % CO_2, the remaining adherent cells consisted of a homogeneous (>99 %) population of bone marrow-derived macrophages (BMM), as determined by staining of cytocentrifuge preparations with the macrophage-specific enzyme alpha-napthyl acetate esterase (see table 5.2). These BMM cells were proliferating as assessed by ^3H incorporation analysis. Day 7 was selected as the optimal day for usage and unless stated otherwise, all procedures using BMM were performed using this time point.

5.4 Stimulation of Cell lines and Bone Marrow-Derived Macrophages

All reasonable precautions were taken to ensure that contamination of experiments with endotoxin / lipopolysaccharide (LPS) did not occur. These included purchase of endotoxin-free recombinant cytokines and antibodies, as well as the usage of sterile plastic tissue culture pipettes and aerosol resistant plugged pipetteman tips. All recombinant cytokines and antibodies were reconstituted according to the manufacturers guidelines in entoxin free PBS / 0.1 % BSA (v/v). Cells were

stimulated as indicated in the figure legends accompanying each figure, with either recombinant cytokines/antibodies or with PBS / 0.1 % BSA (v/v) for negative controls.

5.5 DNA Methodology

5.5.1 Transformation of bacterial host cells with plasmid DNA

Plasmid constructs were obtained from the sources indicated in tables 4.1 and 4.2, and commercially available DH5α competent cells were transformed with the appropriate plasmid construct. Briefly, DH5α cells were removed from -70 °C and thawed on ice. 50 µl aliquots of thawed cells were dispensed into pre-chilled Falcon 2059 tubes, 1 µl of plasmid construct DNA (10-50 ng/µl) added and the mixture very gently swirled, before incubating on ice for 30 minutes. The cells were then heat shocked at 42 °C for exactly 45 seconds, returned to ice for 2 minutes and then 90 µl of Luria broth added. This mixture was then shaken at 225 rpm for 45 minutes at 37 °C, to permit sufficient time for the ampicillin resistance gene to be expressed. 100 µl and 50 µl of transformed cells were then spread onto prewarmed LB-Amp plates (6.5 g of Bactoagar in 400 ml Luria Broth, mixed and autoclaved, allowed to cool to ~37 °C. The agar mix was then supplemented with 100 µg/ml ampicillin and 10-20 ml poured into 10 cm petri dishes. Plates were then allowed to set completely). The spread plates were allowed to stand for 5-10 minutes at room temperature, then inverted and incubated overnight at 37 °C.

5.5.2 Growth of plasmids

A 25 ml sterile universal container containing 5 ml of Luria Broth plus ampicillin (100 µg/ml) was innoculated with a single colony of cells (from LB-AMP plates prepared in section 5.5.1) using a sterile innoculating loop. This was then incubated for 8 hours at 37 °C with shaking at 225 rpm. This starter culture was then added to 100 ml of Luria Broth containing ampicillin (100 µg/ml) in a 1 litre conical flask and incubated overnight at 37 °C with shaking at 225 rpm.

5.5.3 Plasmid preparation

Plasmids were prepared using the commercially available QIAGEN midi or maxi plasmid kits, according to the manufacturer's instructions; -briefly as follows. Bacterial suspensions (derived as in section 5.5.2) were transferred to 250 ml centrifuge bottles (Sorvall instruments) and spun at 3000 rpm for 5 minutes in a GS-3 rotor, using a Sorvall RC-5B superspeed centrifuge. The supernatants were discarded and any residual liquid drained off briefly by inversion. The bacterial pellets were then resuspended in either 4 ml (midi) or 10 ml (maxi) of P1 resuspension buffer (50 mM Tris.HCl, 10 mM EDTA pH 8.0, 100 μg/ml RNase A). Once the pellet was suspended in P1, the bottles were gently rocked on an orbital shaker to allow complete resuspension of the bacterial pellet. The suspension was then transferred to 50 ml centrifuge tubes (Sorvall instruments) and either 4 ml (midi) or 10 ml (maxi) of P2 lysis buffer (200 mM NaOH, 1 % SDS) added. Tubes were immediately capped, gently inverted 4-6 times to mix and incubated at room temperature for 5 min. 4 ml (midi) or 10 ml (maxi) of pre-chilled P3 neutralisation buffer (3 M KAc, pH 5.5) was then added, mixed gently by inverting 5-6 times and incubated on ice for 15 minutes (midi) or 20 minutes (maxi). The tubes were then spun for 30 minutes in an SS-34 rotor at 10000 rpm. The supernatant was then carefully decanted, through a double layered surgical gauze swab to remove particulate material and prevent subsequent blocking of QIAGEN columns. During the above spin, QIAGEN columns were equilibrated with buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % ethanol, pH 7.0, 0.15 % Triton X-100)- 4 ml QBT for tip 100 (midi) or 10 ml QBT for tip 500 (maxi). The filtered supernatant was then applied to the column and allowed to drain through by gravity flow. The column was then washed with 2 x 10 ml (midi) or 2 x 30 ml (maxi) of QC wash buffer (1 M NaCl, 50 mM Tris.HCl, 15 % ethanol, pH 7.0). The DNA was eluted from the column with either 5 ml (midi) or 15 ml (maxi) of QF buffer (1.25 M NaCl, 50 mM Tris.HCl, 15 % ethanol, pH 8.5). The DNA was then precipitated with 0.7 volumes of isopropanol and centrifuged at 10000 rpm at 4 °C for 30 minutes as described above. The supernatant was carefully discarded and the pellet resuspended in 300 μl of distilled water. This was transferred to a 1.5 ml microfuge tube and the DNA re-precipitated with 0.1 volume of 3 M Sodium acetate (pH 5.2) and 2.5 volumes of ethanol and spun at 13500 rpm in a MSE bench-top microfuge, at room temperature for 15 minutes. The pellet was washed carefully with 500 μl of 70

% ethanol and respun at 13500 rpm for 5 minutes. The supernatent was discarded and the pellet was then air-dried for 10 minutes and resuspended in a suitable quantity of sterile distilled water. The amount of DNA present was then quantified as follows. 495 µl of distilled water was mixed with 5 µl of plasmid DNA and the optical density measured at 260 nm and 280 nm, using a Beckman spectrophotometer. A single OD unit at 260 nm (OD_{260} = 1) is equivalent to 50 ug/ml of double stranded DNA, thus allowing the concentration of DNA present in samples to be determined with accuracy. Plasmid DNA was stored at -20 °C before usage.

5.5.4 Digestion of plasmid DNA with restriction enzymes

20-50 µg of plasmid DNA was digested in a total reaction volume of less than 100 µl, as follows. The appropriate amount of DNA to be digested was added to a 1.5 ml screw cap microfuge tube. 0.1 volume (of the total reaction volume) of the appropriate 10 x React™ buffer was then added. Dependent upon which restriction enzyme(s) were selected, the React™ buffer was chosen to give the optimal conditions for the selected enzyme. Where two different restriction enzymes were used, a compromise was reached and a buffer chosen that enabled the highest possible efficiency for both enzymes. If this was not possible, then the One-Phor-all plus™ buffer (Pharmacia, UK) was used instead. The restriction enzyme(s) was then added, making sure the volume did not exceed 0.1 volume of the final reaction volume in order to prevent inhibition of the reaction by glycerol in the enzymes. The reaction mix was then made up to the final desired reaction volume with distilled water and incubated at 37 °C for 60 minutes. Reactions were then either used immediately for downstream applications or were stored at -20 °C until use.

5.5.5 Agarose gel electrophoresis of DNA

The following protocol was used for the routine analysis of DNA derived from plasmid preparations, restriction enzyme digestion of plasmid DNA, quantification of DNA probe fragments and visualisation of products generated by polymerase chain reaction (PCR);- as follows. DNA samples were resolved on a non-denaturing agarose gel. 1-2 % agarose w/v was added to a conical flask and made up to a final volume of either 75 ml for small casting trays or 150 ml for large ones, with distilled water. The

agarose was melted for 2-3 minutes in a standard 750 W microwave, 50 x TAE buffer was then added to give a final working concentration of 1 x TAE and 2.5 µl or 5 µl (small and large gels respectively) of ethidium bromide (10 mg/ml) added. The agarose was then allowed to cool to the point at which it could be safely hand held, before pouring into casting trays. Gels were allowed to set for 30-60 minutes before use). DNA samples were added to 2.5 µl of DNA gel loading buffer (see section 4.15.1) and the volume made up to 10 µl with distilled water (for larger loading volumes the quantities were adjusted accordingly). The DNA was separated by running the gels at a constant 90 volts. The gels were then checked after 1-2 hours by visualisation of the ethidium bromide stained DNA bands on a UV transilluminator (λ=312 nm). The gel image was then recorded using an Appligene imager (Appligene Oncor, UK). For derivation of probe fragments the appropriate band(s) was excised using a sterile scalpel at this point. For Southern blotting of DNA (see section 5.7), the gel was image was recorded alongside a fluorescent ruler and then excess agarose trimmed off prior to blotting.

5.5.6 Isolation of probe fragments for generating radiolabelled probes

Plasmid DNA encoding the selected DNA probe was digested with the appropriate restriction enzymes (see section 5.5.4), resolved by agarose gel electrophoresis and the appropriately sized band of DNA excised from the gel with a sterile scalpel (see section 5.5.5). The gel fragment was collected in a 1.5 ml screw cap tube and the weight of agarose measured. The agarose was removed and the DNA eluted, using the QIAquick gel extraction kit (Qiagen). Briefly, 3 volumes (0.1 g of agarose = approximately 100 µl = 1 volume) of extraction buffer were added to the excised band. This was heated at 55 °C for 10 minutes and the tube inverted every 2 minutes to ensure complete dissolution. This solution was loaded onto a QIAquick spin column and spun at 13500 rpm for 1 min. The flow-through was discarded and the column washed with 750 µl of wash buffer by spinning at 13500 rpm for 1 min. The column was then spun again for an additional minute to remove traces of wash buffer. The DNA was eluted from the column by placing in a clean 1.5 ml eppendorf and adding 30 µl of TE, pH 8.0 to the centre of the spin column. This was left for 1 minute, before the column was spun for 1 minute at 13500 rpm and the DNA

collected. The eluted DNA was visualised and quantified by agarose gel electrophoresis of 2 μl of DNA on a 1 % non-denaturing agarose gel (see section 5.5.5) and comparison against a known quantity of low DNA mass ladder run on the same gel.

5.6 RNA Methodology

5.6.1 RNase free environment

In order to maintain the integrity of RNA samples during isolation, storage and subsequent downstream handling, stringent precautions were taken to prevent degradation by RNases. Disposable sterile plastic pipettes, plugged sterile nuclease-free aerosol resistant pipetteman tips and disposable sterile plastic tubes were used for handling of RNA samples. All such plasticware, as well as chemical reagents were designated exclusively for use with RNA. Bench top surfaces and non-disposable equipment were wiped with RNase-Zap (Invitrogen, Netherlands), a commercially available solution for inhibiting RNases. DEPC treated sterile distilled water was used for making up RNA sample loading buffer, ethidium bromide and for resuspension of RNA samples.

5.6.2 Isolation of total RNA

Cells were stimulated with appropriate growth factors (or PBS / 0.1 BSA (v/v)) and total RNA was then isolated using TRIzol™ (Gibco Life Technologies, UK), a commercially available acid guanidinium thiocyanate-phenol-chloroform based extraction mixture (Chomcyznski *et al* 1987), according to the manufacturer's instructions. Briefly as follows. For RNA isolation from adherent cells in 75 cm^2 flasks, growth medium was removed and 10 ml of Trizol™ added. For isolation of RNA from cells grown in suspension, the cells were spun down at 1000 rpm, the growth medium removed and 10 mls of TRIzol™ added. RNA from adherently and suspension grown cells was isolated in an identical way from this point. The flask was swirled (or the tube gently mixed) to ensure complete coverage of the full cell surface area with TRIzol™ and then left for 5 minutes at room temperature, with occasional swirling of flasks (or mixing of tubes) every 2 minutes. The solution was then pipetted

several times, using an automatic pipette aid, to ensure complete cell lysis. The solution was then transferred to a 15 ml "snap cap" Falcon 2059 tube and 2 ml of chloroform added. Tubes were shaken vigorously for 15 seconds, allowed to stand at room temperature for 2-3 minutes and then spun in a Sorvall RC-5B supercentrifuge at 3000 rpm for 15 minutes using HB4 or HB6 swing-out rotors. The resulting aqueous layer was then promptly removed to a fresh 15 ml tube and 5 ml of isopropanol added. The solution was mixed by inversion and left to stand at room temperature for 10 minutes, after which it was spun at 3000 rpm for 10 minutes. The isopropanol supernatant was discarded and the pellet resuspended in 10 ml of 70 % ethanol, to wash the RNA. Tubes were spun at 3000 rpm for 5 minutes, the ethanol wash discarded and the tubes inverted on the bench for 10 minutes to drain off excess ethanol, before air drying the pellets to remove all remaining ethanol. The RNA was resuspended in 50-200 µl of DEPC treated water, and transferred to sterile 1.5 ml screw cap tubes. The RNA was then quantified by adding 5 µl of RNA to 495 µl of distilled water and measuring the OD_{260} nm (as in section 5.5.3, except that an OD_{260} of 1 is equivalent to 40 µg/ml for RNA). RNA samples were stored at -20 °C until use.

5.6.3 Isolation of PolyA$^+$ mRNA

PolyA$^+$ mRNA was isolated from snap-frozen eye and leg muscle tissue samples or from frozen cell suspensions of peritoneal cavity cells and bone marrow cells derived from either wild type or GM-CSF transgenic mice, using the commercially available Micro-FastTrack™ kit (Invitrogen). Briefly as follows, a mortar and pestle was cleaned with RNase-Zap, placed on dry ice and a piece of snap-frozen tissue was placed in the mortar. The tissue was then ground in liquid nitrogen, before transferring to a clean RNase-free tube. The tissue was then resuspended in 1 ml of lysis buffer, preheated to 45 °C. For frozen cell suspensions the cell pellet was resuspended in 5 ml of lysis buffer and incubated at 45 °C until thawed, before dispersing into 5 x 1 ml aliquots. The cell suspension was then homogenised by passing through a syringe fitted with an 18-21 gauge needle. This was repeated until the entire suspension could be drawn up into the syringe. The mRNA isolation protocol was then followed according to the manufacturer's instructions. The PolyA$^+$ mRNA was eluted in a final volume of 10 µl of elution buffer. 5 µl of mRNA was

then Northern blotted and the membrane hybridised, as described in sections 5.6.4, 5.7 and 5.8.

5.6.4 Agarose gel electrophoresis of RNA

Total RNA was separated and visualised in a similar fashion to that used for DNA samples, the main difference being the usage of denaturing conditions. Total RNA was isolated and quantified (see section 5.6.2.) and 20 µg of RNA was added to sterile 1.5 ml screw cap tubes and freeze-dried in a speedivac. The pellet was then resuspended in 20 µl of RNA sample loading buffer (see section 4.15.2), denatured in a boiling water bath for 5 minutes and samples placed on ice for 2 minutes to prevent renaturation of RNA strands. Tubes were briefly spun in a microfuge and 1 µl of ethidium bromide added (10 mg/ml stock). Samples were loaded and resolved on a denaturing agarose gel (1.4 % w/v agarose, 1 x MOPS buffer, 2.2 M formaldehyde and made up to 150 ml with sterile distilled water). Gels were run at a constant 90 volts, until the Bromophenol blue band had migrated almost 2/3 of the distance of the gel (approximately 3 hours). At this time the integrity of the RNA was checked under UV transillumination and the gel image recorded alongside a fluorescent ruler to allow subsequent sizing of RNA species (as described in section 5.5.5). Gels were then trimmed of excess agarose and Northern blotted (see section 5.7).

5.7 Southern and Northern Blotting of DNA and RNA

DNA and RNA resolved by agarose gel electrophoresis can be transferred to nitrocellulose membranes by capillary transfer. For both Southern and Northern blotting the procedure is essentially the same (except for the use of RNase free solutions). Briefly, a glass tank was partially filled with 500 ml of 10 x SSC and a rectangular piece of glass was raised above the tank, to form a platform. A larger rectangular piece of Whatman 3MM blotting paper was then soaked in 10 x SSC and placed on the glass platform, with the ends of the paper in the tank of SSC thus forming a wick. A second piece of SSC soaked 3MM paper, the same size as the glass, was then placed on top. The agarose gel was then placed with the open wells face down on the platform. A sheet of Hybond-N membrane (Amersham, UK) cut to the same size as the gel was soaked briefly in sterile distilled water, followed by 10 x SSC (RNase free for RNA gels) and then layered over the gel. Clean, redundant strips of

autoradiographic film were then used to cover the exposed platform area around the gel, to prevent bypass of the paper wick by subsequent layers. The membrane was then layered with 2 membrane sized pieces of 3MM paper soaked in SSC and any air excluded from the layers by gently rolling with a 1/2 size plastic pipette. 3-4 layers of paper towelling, each layer 6 towels thick and arranged in a perpendicular direction to the previous layer, were then placed on top. Finally a sheet of glass was used as a platform for the addition of a moderately heavy weight and the gels blotted overnight. Following blotting the membranes were briefly soaked in nuclease-free 10 x SSC to remove any pieces of agarose. The RNA was then fixed on to the membranes by UV crosslinking, using a UV Stratalinker 1800 (Stratagene). Membranes were wrapped in Saranwrap and stored at 4 °C prior to use.

5.8 Probing of Blots with Radiolabelled Probes

Essentially the same procedures were used for probing of both Southern and Northern blots.

5.8.1 Preparation of radiolabelled probes

Probe fragments generated by restriction enzyme digestion were isolated (see section 5.5.6) and radiolabelled using a commercially available random priming kit, Ready-to-Go (Pharmacia, UK), according to the manufacturer's instructions. Briefly, 25-50 ng denatured DNA probe fragment was added to a rehydrated Ready-to-Go tube. 5 µl of [α-^{32}P]-dCTP (3000 Ci/mmol) was added and the reaction incubated at 37 °C for 15 minutes. Unincorporated ^{32}P-labelled nucleotides were separated from the radiolabelled probe by passing the reaction through a NICK column (Pharmacia), according to the manufacturer's instructions. The radiolabelled probe was then denatured by heating in a boiling water bath for 2-3 minutes and placed on ice for 2 minutes prior to use.

5.8.2 Pre-hybridisation and hybridisation conditions for Northern and Southern blots.

Pre-hybridisation and hybridisation of membranes (see section 5.7 for generation) were both carried out at 65 °C, using 0.25 M Na_2HPO_4 / 7 % SDS, pH 7.0 as both pre-hybridisation and hybridisation buffers. These steps were performed using

hybridisation bottles and carried out in a rotary hybridisation oven (Hybaid, UK). Briefly, membranes were layered on top of hybridisation mesh (Hybaid) and then rolled up and placed in the bottles, with up to 4 blots per bottle. 10-20 ml of pre-hybridisation fluid was then added and the blots incubated at 65 °C for at least 1 hour. Following pre-hybridisation the denatured radiolabelled probe was added and hybridisation continued overnight at 65 °C.

5.8.3 Washing of blots and exposure to X-ray film

Membranes were washed once in 2 x SSC / 0.1 % SDS for 30 min at room temperature, followed by 20 min at 65 °C with fresh solution and then with 0.5 x SSC / 0.1 % SDS for 30 min at 65 °C. The membranes were then monitored and additional washing performed if required (20 min at 65°C with 1 x SSC / 0.1% SDS and finally if necessary, once for 10 min with 0.01 x SSC / 0.1 % SDS at 65 °C). After washing the membranes were wrapped in Saranwrap and transferred to an exposure cassette incorporating an intensifying screen. Membranes were exposed to Kodak X-ray film (X OMAT-AR) at -70 °C for 4 hours initially (1 hour for Southerns), then for longer periods (overnight if needed or for up to 7 days as appropriate).

5.8.4 Stripping of blots

Blots were stripped to remove bound radiolabelled probes, by boiling the membranes in 300-500 ml of 0.1 % SDS for 10 min. Membranes could then be used in further experimentation.

5.8.5 Scanning densitometric analysis of blots

Northern and Southern blots were scanned using a flat bed laser densitometer and analysed using the Quantity One software program (both purchased from Protein Databases Inc., NY, USA).

5.9 Protein Detection

5.9.1 Preparation of cell conditioned medium

Bone marrow-derived macrophages were grown for 7 days in 15 ml of α-MEM conditioned medium supplemented with 25 % DHS and 20 % L929 CM, as described in section 5.3. Cells were then either treated with recombinant murine GM-CSF at a concentration of 10 ng/ml, or with PBS / 0.1 % BSA (v/v) for controls. Medium was collected after 12, 24, 48 and 72 hours of treatment.

5.9.2 Purification of MIP-1α from bone marrow-derived macrophage cell conditioned medium

Bone marrow-derived macrophages were grown and treated as described in section 5.9.1. Cell conditioned medium was removed after 12, 24, 48 and 72 hours. 10 ml of each sample of cell conditioned medium was then purified using a combination of heparin and blue sepharose affinity chromatography, followed by reverse phase high performance liquid chromatography (HPLC). This purification of MIP-1α protein was performed by Dr. J. deBono, whom also performed the Western blot shown in figure 6.3.6. His assistance with this purification is very gratefully acknowledged.

5.9.3 SDS polyacrylamide gel electrophoresis

SDS Polyacrylamide gel electrophoresis was performed using a minigel system. 17.5 % polyacrylamide gels were prepared using a commercially available acrylamide / bis-acrylamide stock solution (30 % w/v acrylamide / 0.8 % w/v bis-acrylamide) (Severn Biotech Ltd.), as detailed in section 4.15.3. This solution was then poured between two mini-gel glass plates. The surface of this was then layered with water saturated isobutanol, to create a smooth interface between stacking and resolving gels (as well as preventing oxidisation of the acrylamide mixture and thus speeding up setting). The gel was left to set at room temperature. A 5 % stacking gel was then prepared in a similar way as previously for the resolving gel (see section 4.15.3), except that a smaller quantity was prepared. The water saturated iso-butanol was poured off and the top of the resolving gel washed 3 x with distilled water. Residual water was removed with a small piece of 3MM paper. The 5 % stacking gel was then poured between the glass plates, on top of the resolving gel and a comb of

the appropriate size inserted to create the loading wells. This was allowed to set at room temperature (approximately 30-60 minutes) and the comb carefully removed. Protein samples were prepared for electrophoresis in 1.5 ml screw cap tubes, by adding 20 µl of sample to 15 µl of SDS/DTT sample loading buffer (see section 4.15.3) and samples reduced and denatured in a boiling water bath for 5 minutes. Samples were immediately loaded onto the gel, along with molecular weight rainbow markers (Amersham) to enable subsequent sizing of bands and to verify transfer of proteins during Western blotting. Electrophoresis of gels was carried out at room temperature in SDS/PAGE running buffer (see section 4.15.3) at 150 V, until the blue dye front was at 4 cm from the bottom of the resolving gel.

5.9.4 Western blotting

Western blotting was then used to analyse the purified MIP-1α content of cell conditioned medium derived from bone marrow-derived macrophages. Proteins separated by SDS/PAGE (see section 5.9.5, above) were transferred to Immobilon-P membrane by the process of semi-dry blotting using an electroblotter (Biogel Electophoresis Systems, UK). Gels were blotted for 60 minutes at a constant current of 8 mA/cm^2 gel area, and was performed as follows. Pieces of Whatman 3MM blotting paper were cut to the same size as the gel to be blotted. Four pieces of paper were soaked in transfer buffer (see section 4.15.3) and placed on the base plate of the electroblotter. A piece of Immobilon-P, also cut to the dimensions of the polyacrylamide gel, was pre-soaked briefly in methanol followed by brief washing in distilled water and was soaked in transfer buffer for 2 minutes before layering on top of the blotting paper. The polyacrylamide gel was then placed on top of the Immobilon-P, followed by 3 pieces of blotting paper soaked in transfer buffer. Any air bubbles trapped in the layers were removed by gentle rolling of the top layer with a plastic pipette. The top plate of the electroblotter was moistened with transfer buffer and the apparatus assembled. The electroblotter was then switched on and run at the conditions mentioned above. After blotting the apparatus was disassembled and the membrane incubated overnight in BLOTTO solution (5 % dried milk in PBS, 0.1 % NP40) at 4 °C, to block non-specific binding sites. The membrane was then washed for 1 hour at room temperature in several changes of fresh BLOTTO. The membrane was then exposed to the primary polyclonal antibody, for 1 hour, at the appropriate

dilution (for goat anti-MIP-1α (R&D systems) usually 1:1000 in 10 ml of BLOTTO). The membrane was washed for 45 minutes in at least 3 changes of BLOTTO, before incubation for 40 - 60 minutes with the secondary anti-goat IgG antibody conjugated to horse radish peroxidase (Sigma), in 10-50 ml BLOTTO. The membrane was then washed in several changes of PBS / 0.1 % tween 20 for 1 hour. Binding of antibody to membranes was visualised using the enhanced chemiluminescence (ECL) kit (Amersham). Briefly, the membrane was removed from the final wash step, excess solution drained off and the membrane was then agitated in combined ECL solutions for 60 seconds. Excess solution was drained off from one corner of the membrane, using a paper towel before the membrane was wrapped in Saranwrap and placed in an exposure cassette. An initial exposure was carried out for 2 minutes, using Fuji RX Medical X-ray film, additional exposures were then carried as appropriate to enable protein bands to be visualised.

5.10 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Methodology

5.10.1 DNase I digestion of total RNA

Total RNA samples were treated with DNase I in order to eliminate the potential for residual DNA contaminating total RNA samples, to act as a template for PCR amplification. Briefly, 40 μg of total RNA was digested with 2 μl of DNase I (10 U/μl stock) in the presence of 50 mM sodium acetate (pH 5.2) and 1 M magnesium chloride and made up to 200 μl with DEPC-water. The reaction was incubated at room temperature for 15 minutes, before heating at 95 °C for 5 minutes and then placing on ice for 10 minutes. The reaction was then extracted twice with phenol / chloroform / isoamyl-alcohol (25:24:1), pH 8.0. The RNA was precipitated with 0.1 volume of 3M NaAC and 2.5 volumes of 100 % ethanol for 60 minutes at -70 °C, before spinning down at 13500 rpm for 15 minutes. The pellet was washed with 500 μl of 70 % ethanol and spun down at 13500 rpm for 10 minutes. After brief air drying the pellet was resuspended in 30 μl of DEPC-water (approximately 1 μg/μl).

5.10.2 RT-PCR methodology I (amplification from total RNA)

The commercially available Gene Amp® RNA PCR Core kit (Perkin Elmer, CA, USA) was used to detect the expression of RNA transcripts from total RNA samples. Total RNA was DNase I digested as described in section 5.10.1. The manufacturer's instructions were followed with minor adjustments. Briefly as follows, reverse transcription reactions were set up as 20 µl reactions from a master mix (5 mM $MgCl_2$, 1 x PCR buffer II, 1 U/ml RNase inhibitor, 2.5 µM Oligo $d(T)_{16}$, 1.75 µM random hexamers). 1 µl of DNase I digested RNA (~1 µg/µl) was added to 10 µl of master mix, mixed by pipetting and then incubated at 70 °C for 5 minutes. Then 8 µl of dNTP mix (1 mM each of dGTP, dATP, dCTP and dTTP) was added, followed by 1 µl of MuLV reverse transcriptase (2.5 U/µl) or 1 µl of DEPC-water. The reaction was then incubated for 10 minutes at room temperature to allow the Oligo $d(T)_{16}$ to be extended by the reverse transcriptase. The reaction tubes were then transferred to a Perkin Elmer Thermal Cycler 480, and incubated at 42 °C for 20 minutes, followed by 5 minutes at 99 °C and 15 minutes at 5 °C. A PCR master mix was then prepared (0.5 mM $MgCl_2$, 1 x PCR buffer II, *AmpliTaq*® DNA polymerase (2.5 U/100 µl)) and 74 µl added to 20 µl of reverse transcription reaction from above. 3 µl each of 5' and 3' primers (200 ng/µl) were then added (see tables 4.3 and 4.4 for identity and sequences). The whole reaction mix was mixed by pipetting and then overlaid with 100 µl of liquid paraffin. Tubes were incubated in a Thermal Cycler for 1 minute at 94 °C and then for 30-35 cycles at the following conditions. A) For amplification of MIP-1α sequences: 1 minute at 94 °C, 2 minutes at 60 °C, 2 minutes at 72 °C, followed by soaking at 4 °C. B) For amplification of CC chemokine receptor sequences: 1 minute at 94 °C, 1 minute at 55 °C, 1 minute 30 seconds at 72 °C, followed by soaking at 4 °C. C) For amplification of IL-5Rα chain fragments: 1 minute at 94 °C, 2 minutes at 56 °C, 3 minutes at 72 °C, followed by soaking at 4 °C. Samples were stored at 4 °C prior to visualisation of 20 µl of amplified DNA product, by agarose gel electrophoresis on a 1 % agarose gel.

5.10.3 RT-PCR methodology II (amplification from polyA$^+$ mRNA)

The Gene Amp® RNA PCR Core kit was again used, this time for the detection of RNA transcripts from polyA$^+$ mRNA. PolyA$^+$ mRNA was derived (as

described in section 5.6.3) and 0.5 µl (eye and leg muscle) or 2 µl (bone marrow or peritoneal cells) of this used as a template for the generation of cDNA, prior to PCR amplification. The basic RT-PCR methodology was exactly as described for total RNA, with the following modifications: 1) the reactions were performed in 10 µl reaction volumes for the reverse transcriptase step and 50 µl for the PCR step, 2) the use of random hexamers was omitted, 3) A single master mix was prepared for the reverse transcriptase step, and then split into 2 tubes prior to the addition of either Wild type or GM-CSF transgenic polyA$^+$ mRNA. The reverse transcriptase step was then carried out as before. 4) The 2 reverse transcriptase reactions were then split into 2 again and a PCR master mix added to each of the 4 tubes. 5) 5' and 3' primers for the detection of either MIP-1α or β-Actin were then added to each reaction as appropriate. 6) Each of the 4 reactions (wildtype or GM-CSF with either MIP-1α or β-Actin primers) was then split into 5 tubes. Tubes were then incubated in a Thermal Cycler for 1 minute at 94 °C and then at the following conditions 1 minute at 94 °C, 2 minutes at 60 °C, 2 minutes at 72 °C. Tubes were removed after 15, 20, 25, 30 and 35 cycles respectively and stored at 4 °C prior to analysis on a 2 % agarose gel.

5.11 Receptor Binding Studies

5.11.1 ^{125}I labelling of MIP-1α protein

Labelling of MIP-1α protein with Na^{125}I (Dupont NEN) using Iodo-gen, a commercially available iodination reagent, was performed by Dr. R.J.B. Nibbs as described previously (Graham *et al* 1993). The assistance of Dr. Nibbs in this labelling and during other aspects of these studies is very gratefully acknowledged.

5.11.2 MIP-1α receptor binding studies on bone marrow-derived macrophages

BMM were grown as described in section 5.3. At day 5 of growth, cells were scraped free and replated at 2 x 10^5 cells in 24 well plates and then incubated at 37 °C for a further 24 h to allow cell re-adherence. After this time the cells were stimulated with 10 ng/ml recombinant murine GM-CSF or with PBS / 0.1 % BSA (v/v) for 16 h. Treatment of plates was staggered at 30 minute intervals, to allow sufficient time to process each plate. After 16 h treatment the plates were washed with PBS and the cells

incubated with 250 µl of binding buffer (α-modified MEM supplemented with 25 % DHS, 20 % L929 CM, 0.2 % sodium azide and 25 mM Hepes (pH 7.4)) containing 4 nM ^{125}I labelled MIP-1α and variable concentrations of unlabelled MIP-1α competitor or PBS, up to a final volume of 260 µl. Cells were then washed 3 times with 2 ml of ice-cold PBS. The amount of bound radioactivity was determined by lysing the washed cells in 0.5 ml 1 % SDS and counting the lysate in a gamma counter (Beckman Gamma 5500B). Each point was assayed in triplicate and experiments performed at least twice. Analysis of binding results was then performed using the LIGAND software program (Munson and Robard 1980).

5.12 Calcium Flux Studies

5.12.1 Calcium flux studies in bone marrow-derived macrophages

BMM were grown as described in section 5.3. Cells were either stimulated on day 7 with 10 ng/ml recombinant murine GM-CSF or with PBS for 16 h. Cells were then washed once with PBS and scraped free into HACM buffer (see section 4.15.5) and spun down at 1000 rpm for 5 minutes. Cells were resuspended at 1 x 10^6 cells/ml and washed twice in HACM buffer. The tubes containing the cell suspensions were then covered in aluminium foil and the light sensitive fluorescent dye Fura-2 AM added to the cell suspension, at a concentration of 2.5 µM. The cell suspension was then incubated at 37 °C for 1 hour. The tubes were gently inverted to mix the suspension after 20 and 40 minutes of incubation. The cells were spun down at 1000 rpm for 5 minutes and washed twice with warmed HACM buffer. Cells were then resuspended at 2 x 10^6 /ml in warmed HACM buffer. Cells were kept at 37 °C and used immediately for fluxing studies, briefly as follows. A spectrophotometer cuvette, containing a magnetic stirrer bar was placed in a Perkin Elmer luminescence spectrophotometer LS50 and 2 ml of cell suspension was aliquoted directly into the cuvette. After 15 seconds 20 µl of $CaCl_2$ (100 mM stock) was added and the cuvette incubated at 37 °C for 2 minutes, with a high level of stirring. The time drive was then started on the spectrophotometer and after 15 seconds, MIP-1α (final concentration 100 ng/ml) was added directly to the cell suspension in the cuvette. Overall changes in

the intensity of FURA-2 AM were then assessed, thus enabling any changes in intracellular calcium mobilisation to be determined.

5.12.2 Calcium flux studies in THP-1 cells

THP-1 cells were grown in RPMI 1640 / 10 % FCS to a concentration of 5 x 10^5 /ml, as described in section 5.2.1. Cells were then stimulated with either 10 ng/ml recombinant human GM-CSF or PBS / 0.1 % BSA (v/v), for 16 h. Cells were spun down and washed once in PBS and then resuspended at 1 x 10^6 cells/ml and washed twice in HACM buffer. Calcium flux studies were then performed exactly as described in section 5.12.1 above.

PART III: RESULTS AND DISCUSSION

CHAPTER 6: RESULTS

6.1 Overview

In order to investigate the potential of haemopoietic growth factors to regulate the expression of MIP-1α, it was first necessary to find a cell type that provided a system suitable for such investigation. As described in section 1.4.1, the macrophage is one of the primary cell types within the stromal microenvironment. It is therefore likely to be an important source of MIP-1α within the bone marrow and in peripheral tissues. Murine 'bone marrow-derived macrophages' (BMM) provide a homogeneous population, previously used by others as a model system for marrow macrophages (Temeles *et al* 1993) and which are suitable for investigations on bone marrow-derived MIP-1α. These cells have also previously been successfully used by our laboratory, in other studies on MIP-1α regulation (Maltman *et al* 1993, 1996). BMM were therefore selected as a model cell system, with which to investigate the regulation of MIP-1α expression by haemopoietic growth factors.

It is widely recognised that a large interacting network functions within the bone marrow to regulate the processes of constitutive and inducible haemopoiesis (see introduction) and current evidence suggests that MIP-1α is a member of this network. At present, little is known about the regulatory factors controlling MIP-1α expression within the bone marrow. Initially therefore, known haemopoietic growth factors with previously identified effects on macrophage functions, were investigated for their ability to modulate MIP-1α expression in BMM.

6.2 Effect of G-CSF, LIF and IL-6 on the Expression of MIP-1α in BMM

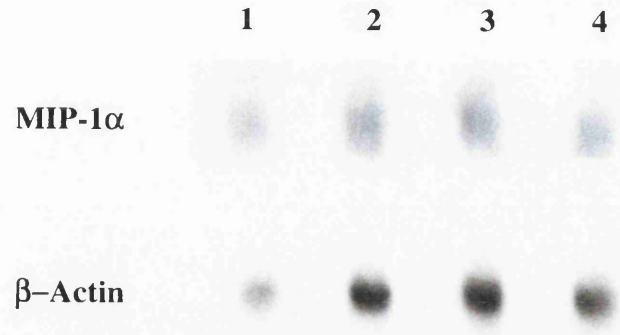
G-CSF, LIF and IL-6 are all important regulators of various facets of haemopoiesis (see introduction). LIF receptors are known to be expressed on both normal and leukaemic macrophages (Hilton *et al* 1988), whilst G-CSF receptors have been reported to be expressed on macrophages (Shieh *et al* 1991), as have receptors for IL-6 (Lotem and Sachs 1987, Shabo *et al* 1988). Their effect on the expression of MIP-1α mRNA in day 7 BMM was therefore investigated. This was performed by Northern blot analysis of total RNA isolated from BMM after 4 h treatment with various concentrations of G-CSF, LIF or IL-6. As shown in Figure 6.1,

Figure 6.1: Northern blot analysis of the effect of G-CSF, IL-6 and LIF on the expression of MIP-1α mRNA in BMM.

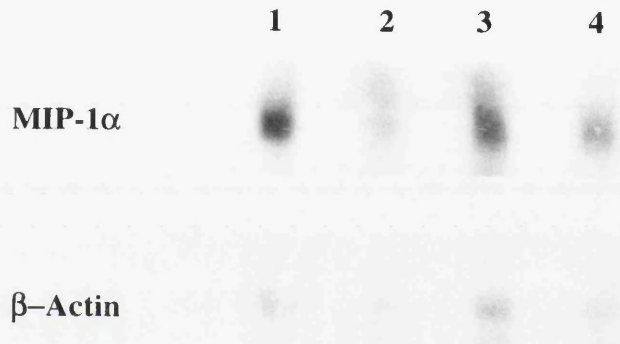
A) Effect of G-CSF. 1) control; 2) 4 h 1 ng/ml; 3) 4 h 10 ng/ml; 4) 4 h 50 ng/ml.
B) Effect of IL-6. 1) control; 2) 4 h 1 ng/ml; 3) 4 h 10 ng/ml; 4) 4 h 50 ng/ml.
C) Effect of LIF. 1) control; 2) 4 h 10 U/ml; 3) 4 h 100 U/ml. Top blot in each panel shows a blot hybridised with a probe for MIP-1α, whilst the bottom blot in each panel shows the same blot, stripped and reprobed for β-Actin. This figure represents a single experiment. The numbers in the table below show the relative levels of MIP-1α mRNA for this experiment, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA.

	Control	**1 ng/ml**	**10 ng/ml**	**50 ng/ml**
G-CSF	1	1.32	1.42	1.28
IL-6	1	0.36	0.53	0.68
	Control	**10 U/ml**	**100 U/ml**	
LIF	1	0.48	0.56	

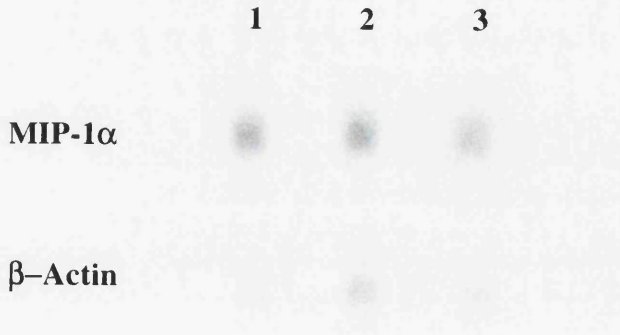
A



B



C



a low basal level of MIP-1α expression was detectable in unstimulated BMM. Addition of G-CSF, LIF or IL-6 had no apparent effect on this level of expression, compared with control cells, as determined by densitometric analysis of Northern blots and normalisation for RNA loading using the house-keeping gene β-Actin. This demonstrated that G-CSF, IL-6 and LIF do not appear to modulate MIP-1α expression in BMM.

6.3. Effect of GM-CSF on the Expression of MIP-1α mRNA in BMM

6.3.1 GM-CSF induces MIP-α expression

The cytokine GM-CSF has been widely studied for its effects on various haemopoietic cell types, particularly those of the granulocyte / macrophage lineages. It was therefore considered a potential candidate for regulation of macrophage-derived MIP-1α expression within the bone marrow. The effects of GM-CSF on MIP-1α mRNA expression in BMM were investigated by Northern blot analysis and as previously, a low level of basal MIP-1α expression was observed. However, following treatment of BMM with recombinant murine GM-CSF (10 ng/ml) for 4 h, this expression appeared to be elevated by approximately 10-fold, as indicated in figure 6.2. Ethidium bromide staining confirmed that loading of RNA in each lane was equal (data not shown). In all future Northern blot analysis, blots were stripped and re-probed with β-Actin to allow normalisation for any loading differences between lanes. The striking level of induction observed in GM-CSF treated BMM, compared to that of control cells, suggested that GM-CSF functions as a modulator of MIP-1α expression and therefore warranted further investigation.

6.3.2 Induction of MIP-α expression by GM-CSF is rapid, but transient

To further assess the effect of GM-CSF on MIP-1α expression, BMM were treated with GM-CSF for various lengths of time and then the levels of MIP-1α mRNA were analysed as previously, by Northern blotting and densitometric analysis. Figure 6.3 shows one such experiment. MIP-1α expression was rapidly induced, by approximately 3-fold compared to control cells, within 1 h following GM-CSF addition (compare lanes 1 and 2). In fact, in some other experiments increased

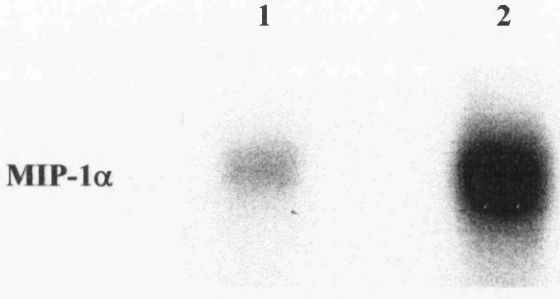


Figure 6.2: Northern blot analysis of the effect of GM-CSF on the expression of MIP-1α mRNA in BMM.

1) control; 2) 4 h 10 ng/ml GM-CSF. Loading levels were confirmed by ethidium bromide staining. This figure represents a single experiment. Scanning densitometric analysis revealed that the level of MIP-1α mRNA in GM-CSF treated cells (lane 2) was increased by approximately 9.63-fold relative to that of control treated cells (lane 1).

Figure 6.3:

This figure represents data for a single experiment. The numbers in the table below show the relative levels of MIP-1α mRNA for this experiment, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in 1 h control treated cells. * = data shown in the figure opposite.

	1 h Control	1 h GM-CSF	2 h GM-CSF	4 h GM-CSF	6 h GM-CSF
	*1.00	*3.34	*3.50	*13.7	*17.4
	1.00	3.64	5.00	22.9	9.03
Average	1.00	3.49	4.25	18.3	13.2

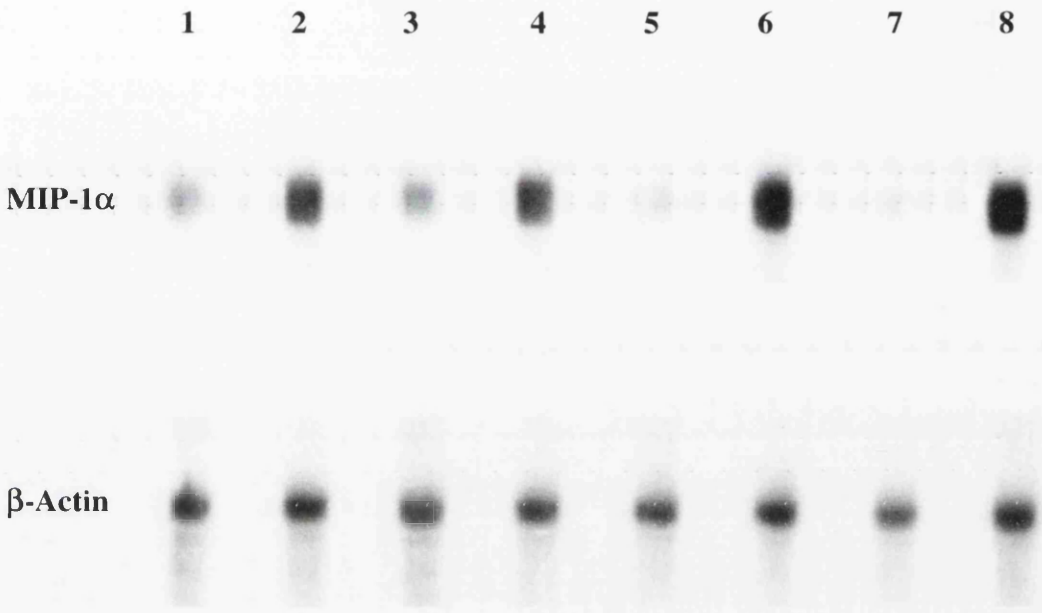


Figure 6.3: Northern blot analysis of the effect of GM-CSF on a time course of MIP-1α expression in BMM.

1) 1 h control; 2) 1 h GM-CSF (10 ng/ml) ; 3) 2 h control; 4) 2 h GM-CSF; 5) 4 h control 6) 4 h GM-CSF; 7) 6 h control; 8) 6 h GM-CSF. Top panel shows a blot hybridised with a probe for MIP-1α, the bottom panel shows the same blot, stripped and reprobed for β-Actin.

expression was visible within 30 minutes of GM-CSF stimulation (data not shown). The level of MIP-1α mRNA increased between 1 and 4 h following GM-CSF stimulation, with up to a 20-fold increase relative to control cells (compare lanes 5 and 6). Comparison of lanes 7 and 8 shows that MIP-1α expression was still elevated after 6 h, though not more so than at 4 h. In most experiments however, this peak was reached 4 h after stimulation with GM-CSF and subsequently declined within 6 h (data not shown). Treatment of BMM with GM-CSF for 4 h gave the most consistent increase in MIP-1α expression.

6.3.3 Induction of MIP-α mRNA expression in response to a titration of GM-CSF concentration

In order to assess the potency of the inductive effect of GM-CSF on MIP-1α mRNA expression, BMM were stimulated with a titration of GM-CSF (1 pg/ml to 10 ng/ml) and the level of MIP-1α mRNA assessed by Northern blot analysis 4 h after addition of GM-CSF. Figure 6.4 shows MIP-1α mRNA expression to be induced in a dose dependent manner, and densitometric analysis demonstrated that as little as 0.1 ng/ml of GM-CSF resulted in approximately a 3-fold increase in the level of MIP-1α mRNA compared to control values (compare lanes 1 and 4). The increase in MIP-1α mRNA appeared to plateau with between 5 and 10 ng/ml GM-CSF, resulting in approximately a 10-fold increase in this experiment (compare lane 1, with lanes 7 and 8). However, it should be noted that the MIP-1α blot is overexposed and the increase in MIP-1α is perhaps therefore likely to be greater than that suggested by the scanning densitometric analysis. As 10 ng/ml appeared to be just beyond the "shoulder" of this plateau and therefore likely to give a full induction, all future experiments were performed using 10 ng/ml GM-CSF for 4h (unless stated otherwise).

6.3.4 Induction of MIP-α mRNA expression is not dependent on factors present in serum or L929 conditioned medium

BMM are grown in the presence of high levels of both serum and M-CSF (in the form of L929 conditioned medium). These cells will enter a quiescent state if deprived of serum for 16 h and absolutely require the presence of a source of M-CSF for their differentiation, continued proliferation and survival (Becker *et al* 1987). In

Figure 6.4:

This figure represents data for a single experiment. The numbers in the table below show the relative levels of MIP-1α mRNA for this experiment, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.

	Concentration of GM-CSF added						
Control	**1 pg/ml**	**10 pg/ml**	**0.1 ng/ml**	**0.5 ng/ml**	**1 ng/ml**	**5 ng/ml**	**10 ng/ml**
1.00	1.10	1.74	3.1	4.4	8.1	11	9.20

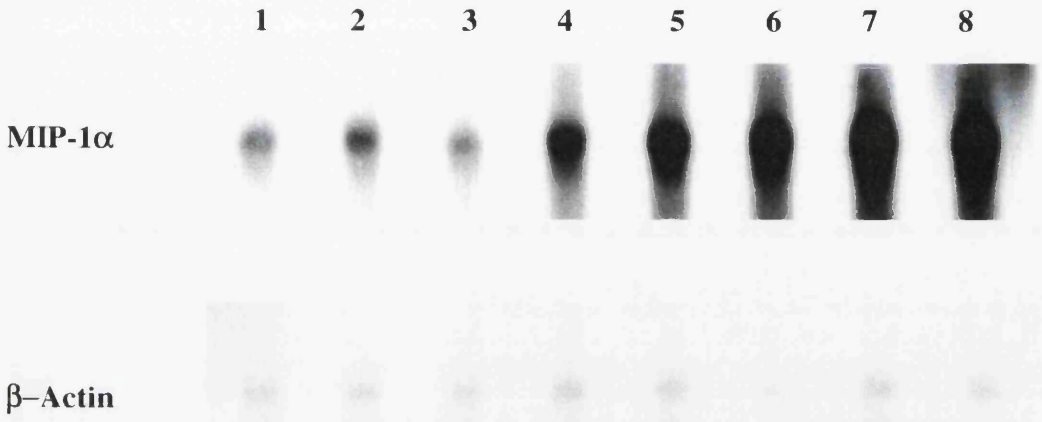


Figure 6.4: Northern blot analysis of the effect of a titration of GM-CSF on MIP-1α mRNA expression in BMM.

BMM were treated for 4 h with the following concentrations of GM-CSF:
1) control; 2) 1 pg/ml; 3) 10 pg/ml; 4) 0.1 ng/ml; 5) 0.5 ng/ml; 6) 1 ng/ml;
7) 5 ng/ml; 8) 10 ng/ml. Top panel shows a blot hybridised with a probe for MIP-1α, the bottom panel shows the same blot, stripped and reprobed for β-Actin.

order to assess whether either serum or L929 conditioned medium (L-929 CM) played a co-operative part in the induction of MIP-1α expression by GM-CSF, cells were stimulated for 4 h with GM-CSF in the presence or absence, of either serum or L-929 CM.

Panel A of figure 6.5 shows that similar levels of MIP-1α mRNA were induced in either the presence, or absence of serum. Densitometric analysis revealed that this induction was approximately 4-fold, in both the presence or absence of serum.

Panel B of figure 6.5 shows that the absence of L-929 CM did not result in the abolition of GM-CSF induced MIP-1α expression. In fact, densitometric analysis revealed that in the absence of L-929 CM the induction of MIP-1α mRNA appeared to increase by approximately 170 %, rising from 11-fold in the presence of L-929 CM to 29-fold in the absence of L-929 CM. This suggests that MIP-1α suppressive factors may be present in the conditioned medium of L-929 cells.

These results demonstrate that GM-CSF does not require the presence of factors present in either serum or L-929 CM, to induce MIP-1α mRNA expression.

6.3.5 GM-CSF directly induces the expression of MIP-1α mRNA

It has previously been demonstrated using neutrophils that LPS is able to synergise with GM-CSF to induce MIP-1α expression, although GM-CSF was unable to induce MIP-1α expression when used as a sole agent (Kasama *et al* 1993). In order to exclude the presence of LPS as far as possible and therefore rule out a role for LPS in the observed induction of MIP-1α expression, BMM were stimulated under essentially LPS-free conditions. To demonstrate that GM-CSF was indispensable for this observed induction, the effects of GM-CSF on MIP-1α expression were assessed in the presence of a GM-CSF specific neutralising antibody. Figure 6.6 shows that as previously, GM-CSF increased the level of MIP-1α mRNA by approximately 10-fold relative to control values. However, in the presence of a GM-CSF neutralising antibody this induction was almost completely abrogated. Densitometric analysis of blots revealed that the basal level of MIP-1α expression did not appear to be significantly different between the control and those cells treated with neutralising antibody. This suggests that there is no autocrine regulation of MIP-1α expression in

Figure 6.5; Northern blot analysis of the effect of GM-CSF on MIP-1α mRNA expression in BMM, in the absence or presence of either serum or L-929 CM.

A) Effect of serum. BMM were treated as follows: 1) control plus serum; 2) 4 h GM-CSF (10 ng/ml) plus serum; 3) control (serum-free); 4) 4 h GM-CSF (serum-free). *B)* Effect of L-929 CM. 1) control plus L-929 CM; 2) 4 h GM-CSF (10 ng/ml) plus L-929 CM; 3) control (L-929 CM-free); 4) 4 h GM-CSF (L-929 CM-free). Top blot in each panel shows a blot hybridised with a probe for MIP-1α, whilst the lower blot in each panel shows the same blot stripped and reprobed for β-Actin. This figure represents single experiments. Tables A and B below, show the relative levels of MIP-1α mRNA for the experiments shown in panels A and B (opposite), as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA.

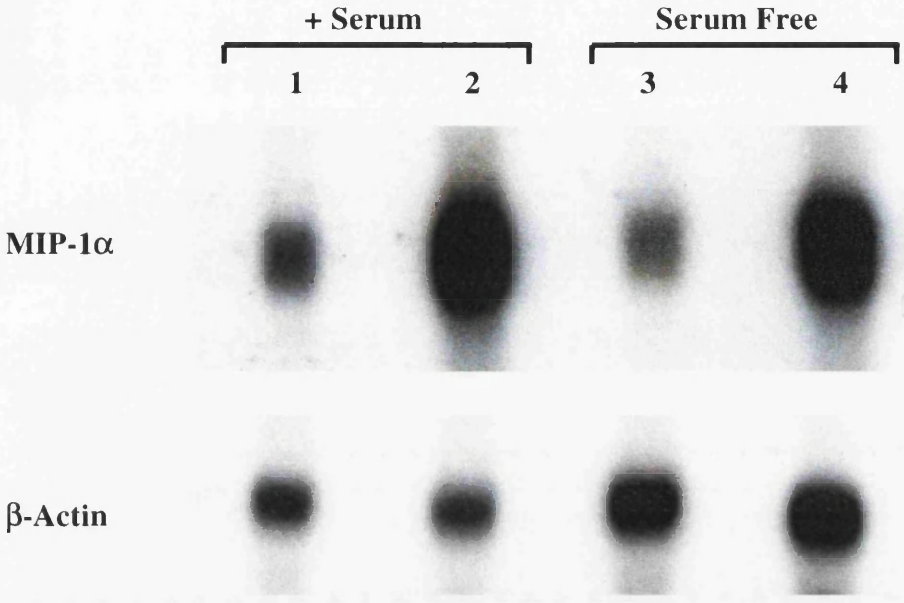
A

Plus Serum		Serum Free	
Control	**GM-CSF**	**Control**	**GM-CSF**
1	4.0	1	3.2

B

Plus L-929 CM		Minus L-929 CM	
Control	**GM-CSF**	**Control**	**GM-CSF**
1	10.7	1	28.7

A


+ Serum
Serum Free
1
2
3
4
MIP-1α
β-Actin

B

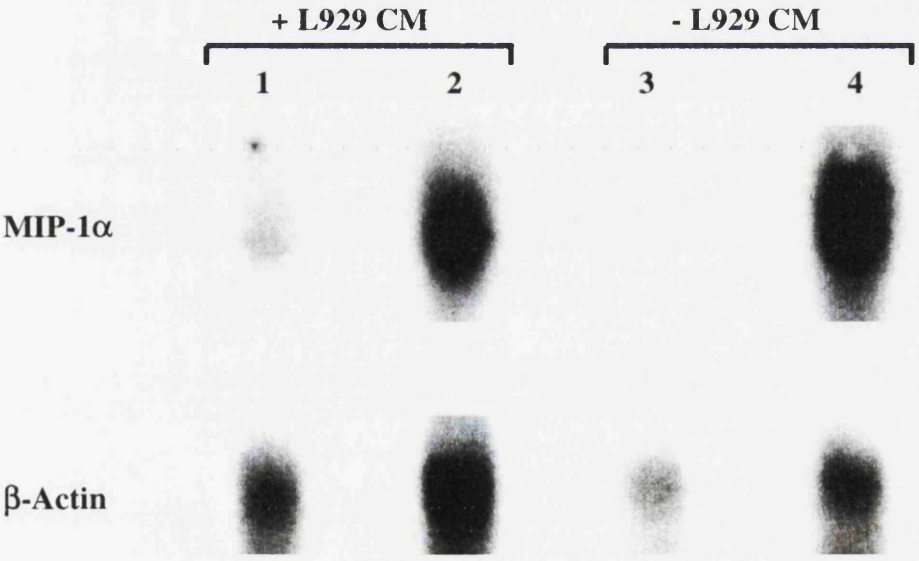

+ L929 CM
- L929 CM
1
2
3
4
MIP-1α
β-Actin

Figure 6.6;

The numbers in the table below show the relative levels of MIP-1α mRNA for these 2 experiments, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.
* = data shown in the figure opposite

	Control	GM-CSF	GM-CSF / Nab	Nab
	*1.00	*23.0	*4.23	*3.41
	1.00	34.0	2.60	0.86
Average	1.00	28.5	3.40	2.14

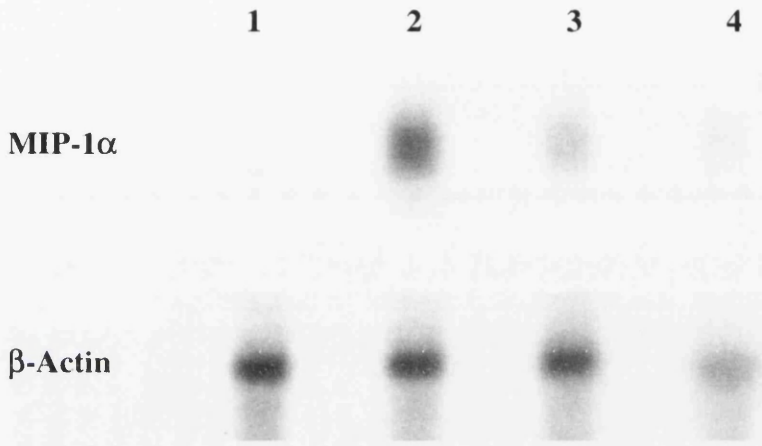


Figure 6.6: Northern blot analysis of the effect of a neutralising GM-CSF antibody on GM-CSF induced MIP-1α mRNA expression in BMM, under LPS-free conditions.

BMM were treated as follows: 1) control; 2) 4 h 10 ng/ml GM-CSF; 3) 10 ng/ml GM-CSF plus neutralising antibody (5 μg/ml); 4) neutralising antibody. Top panel shows a blot hybridised with a probe for MIP-1α, the bottom panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of 2 separate experiments.

macrophages, by GM-CSF.

These results suggest that GM-CSF directly induces the expression of MIP-1α mRNA and because these experiments were performed using conditions and reagents known to be essentially LPS-free, it is highly unlikely that GM-CSF is acting in synergy with LPS.

6.3.6 GM-CSF induces MIP-1α protein expression in BMM

To assess whether increases in MIP-1α expression at mRNA level were also reflected by increases in MIP-1α protein expression, BMM were either treated with GM-CSF (10 ng/ml) or PBS / 0.1 % BSA (v/v), and conditioned medium collected after various lengths of time. The samples were collected later than for total RNA, to allow sufficient time for MIP-1α mRNA to be translated to protein and to allow time for the levels of protein to accumulate in the macrophage growth medium to a level at which they could be detected. The content of MIP-1α protein in these BMM supernatants was then measured by Western blot analysis. In initial experiments, no MIP-1α protein could be detected in supernatants from either control or GM-CSF treated BMM, although a recombinant MIP-1α control was detectable. These observations suggested either that the level of MIP-1α protein in these cells was too low to detect by Western blot analysis, or that GM-CSF stimulation does not result in an increase in MIP-1α protein expression.

It has previously been demonstrated that the level of MIP-1α protein in unstimulated BMM is extremely low and often undetectable by Western blot analysis (Maltman *et al* 1993). Therefore, in order to assess whether MIP-1α protein expression was induced in BMM by GM-CSF, MIP-1α present in supernatants from control and GM-CSF stimulated BMM was purified by a combination of heparin and blue sepharose affinity chromatography, followed by reverse phase HPLC. This purification simultaneously also resulted in approximately a 100-fold concentration of protein. The level of purified MIP-1α protein in these supernatants was then assessed by Western blotting analysis. Figure 6.7 shows that MIP-1α protein was undetectable in unstimulated BMM over the time course of the experiment, but that treatment with GM-CSF (10 ng/ml) resulted in an increased level of MIP-1α protein expression. This increase was just visible 24 h after addition of GM-CSF and increased steadily with

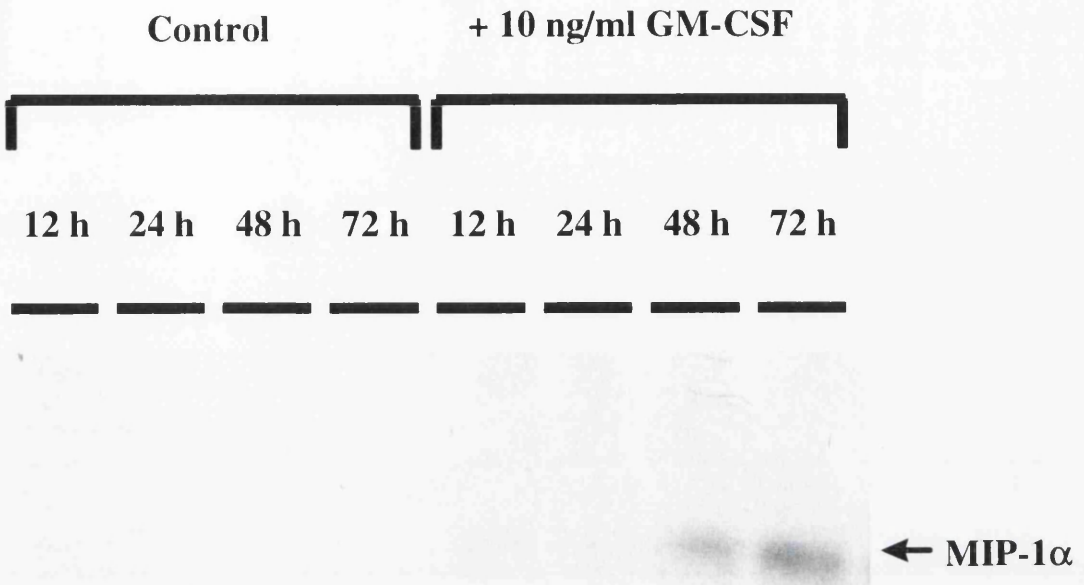


Figure 6.7: Western blot analysis of the effect of GM-CSF on MIP-1α protein expression in BMM.

BMM cell conditioned medium was collected after the appropriate time as denoted at the top of each lane. BMM were treated as follows: PBS /0.1 % BSA control (left hand 4 lanes) or GM-CSF (10 ng/ml) (right hand 4 lanes). Proteins were resolved by SDS/PAGE on a 17.5 % gel. MIP-1α protein was detected using a murine anti-MIP-1α antibody and ECL reagents, as detailed in the Materials and Methods.

This figure represents data for a single experiment and both the protein purification and the Western blot analysis were performed by Dr. Johann de Bono, as detailed in the materials and methods. The reproduction of his work here, is very gratefully acknowledged.

time up to 72 h. This data suggests that whilst the level of MIP-1α protein in BMM is very low, a finding in accordance with previous reports from our laboratory (Maltman *et al* 1993), the level of MIP-1α protein does increase following GM-CSF treatment. This demonstrates that induction of MIP-1α expression observed at an mRNA level, is also reflected at the level of MIP-1α protein expression and suggests that the induction of MIP-1α by GM-CSF may be physiologically important.

6.4 Effect of IL-3 on MIP-1α mRNA Expression in BMM

GM-CSF is a member of a cytokine family and exhibits some functional overlap with IL-3. It is speculated that this is the result of the shared usage of a receptor component, the common β subunit (or βc), found as a component of both GM-CSF and IL-3 heterodimeric receptors (see introduction). Moreover, IL-3 has previously been reported, along with GM-CSF, to affect bone marrow-derived macrophage functions (Takamatsu *et al* 1996). It was therefore speculated that IL-3 might also exert an inductive effect on MIP-1α expression in BMM, in a similar fashion to that of GM-CSF.

6.4.1 Titration of the inductive effect of IL-3 on MIP-1α mRNA expression

To assess the ability of IL-3 to regulate MIP-1α mRNA expression, BMM were treated with a titration of IL-3 (1 pg/ml to 10 ng/ml) for 4 h. Panel A of figure 6.8 shows MIP-1α mRNA expression to be increased in a dose dependent manner, and densitometric analysis revealed that concentrations as low as 0.1 to 1 ng/ml of IL-3 were sufficient to induce substantial expression of MIP-1α mRNA. As with GM-CSF, the expression of MIP-1α plateaued with between 5 ng/ml and 10 ng/ml of IL-3, resulting in approximately a 20 to 25-fold increase in the levels of MIP-1α mRNA relative to control values. A time course of MIP-1α expression in response to IL-3 (10 ng/ml), also revealed a similar time course to that seen GM-CSF (data not shown). As a dose of 10 ng/ml appeared to be just beyond the shoulder of this plateau, all future stimulations using IL-3 were performed using a dose of 10 ng/ml for 4 h. These results indicate that in addition to GM-CSF, IL-3 is also an inducer of MIP-1α expression.

Panel B of figure 6.8 shows that treatment of BMM with IL-3 increased the level of MIP-1α mRNA by approximately 23-fold relative to control values, but that

Figure 6.8: Northern blot analysis of the effect of IL-3 on MIP-1α mRNA expression in BMM.

A) Effect of a titration of IL-3 on MIP-1α mRNA expression. BMM were treated for 4 h with the following concentrations of IL-3: 1) control; 2) 1 pg/ml; 3) 10 pg/ml; 4) 0.1 ng/ml; 5) 0.5 ng/ml; 6) 1 ng/ml; 7) 5 ng/ml; 8) 10 ng/ml. *B)* Effect of a neutralising IL-3 antibody on IL-3 induced MIP-1α mRNA expression. BMM were treated for 4 h as follows: 1) control; 2) 10 ng/ml IL-3; 3) 10 ng/ml IL-3 plus neutralising antibody (5 μg/ml); 4) neutralising antibody. Top blot in each panel shows a blot hybridised with a probe for MIP-1α, the bottom blot in each panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of a single, and 2 separate experiments respectively. Table A below shows the relative levels of mRNA for MIP-1α (as shown in panel A, opposite) and calculated from the data obtained by scanning densitometric analysis and normalisation against the calculated level of β-Actin mRNA. Table B shows the relative levels of mRNA for MIP-1α, calculated from 2 separate experiments (* = data for experiment shown in panel B, opposite).

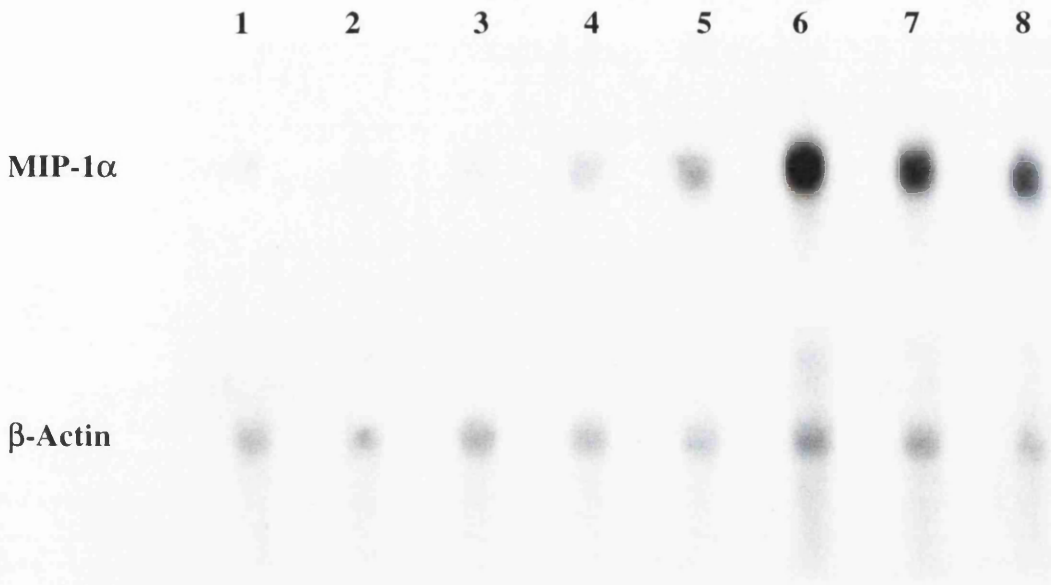
A

	Concentration of IL-3						
Control	**1 pg/ml**	**10 pg/ml**	**0.1 ng/ml**	**0.5 ng/ml**	**1 ng/ml**	**5ng/ml**	**10ng/ml**
1.00	1.20	1.00	2.80	10.0	25.0	18.0	24.0

B

	Control	**IL-3**	**IL-3/NAb**	**NAb**
	*1.00	*23.2	*0.55	*0.53
	1.00	24.8	6.40	3.16
Average	1.00	24.0	3.48	3.69

A



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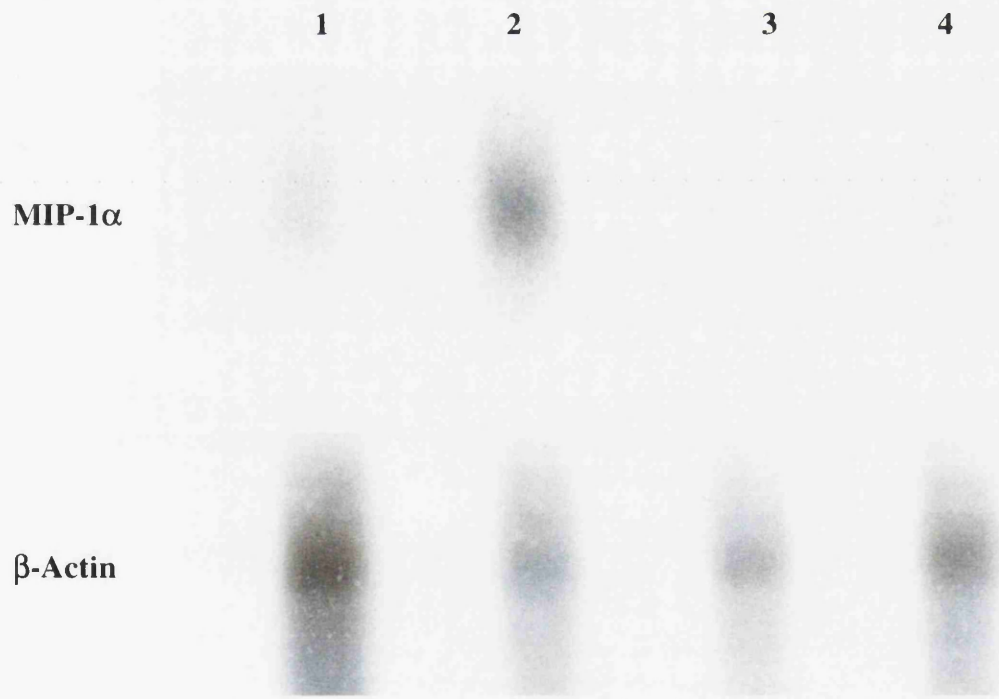


in the presence of an IL-3 neutralising antibody this induction was reduced to the same level as that of the control cells. As with GM-CSF, densitometric analysis of blots showed that the basal level of MIP-1α expression appeared to be similar in both control and IL-3 neutralising antibody treated cells, suggesting that there is no involvement of autocrine IL-3 in regulating the overall expression of MIP-1α in macrophages. In summary, these data suggest that, like GM-CSF, IL-3 directly induces the expression of MIP-1α mRNA. This experiment was performed using conditions and reagents known to be essentially LPS-free and IL-3 is therefore unlikely to be acting in synergy with LPS.

6.5 Effect of IL-5 on MIP-1α mRNA Expression

IL-5 is also a member of the same small subfamily of cytokines, comprised of GM-CSF and IL-3, and in addition shares the βc component in its receptor. The ability of IL-5 to regulate MIP-1α expression was therefore investigated.

6.5.1 IL-5 does not induce expression of MIP-1α mRNA in BMM

BMM were treated with a titration of IL-5 (1 pg/ml to 10 ng/ml) and the level of MIP-1α mRNA analysed after 4 h. Figure 6.9 shows a Northern blot of one such experiment. Densitometric analysis revealed that the level of MIP-1α expression was not altered following treatment of BMM with IL-5, at any of the concentrations tested. This data suggests either that IL-5 has no effects on the expression of MIP-1α, at the concentrations tested, or that BMM do not have the capacity to respond to IL-5. The observation that no increase in MIPα expression occurred in the presence of IL-5, also suggests that LPS was not present in these experiments and confirms that this system is very clean with respect to the potential presence of LPS.

6.5.2 BMM do not express mRNA for the IL-5Rα chain

To attempt to explain the inability of BMM to respond to IL-5, expression of the IL-5 specific α chain receptor subunit (IL-5Rα) was examined in BMM by Northern blot analysis and RT-PCR analysis. No expression of IL-5Rα chain was detectable in BMM by Northern blot analysis (data not shown). Figure 6.10 shows that IL-5Rα chain expression was also undetectable in BMM by RT-PCR analysis (lane 3).

Figure 6.9:

The numbers in the table below show the relative levels of MIP-1α mRNA for these 3 experiments, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.
* = data shown in the figure opposite

		Concentration of IL-5						
	Control	**1 pg/ml**	**10 pg/ml**	**0.1ng/ml**	**0.5ng/ml**	**1 ng/ml**	**5 ng/ml**	**10 ng/ml**
	*1.0	*1.41	*1.10	*1.44	*0.60	*0.98	*0.90	*2.10
	1.0	1.54	0.77	1.09	1.37	2.20	0.90	1.50
	1.0	0.60	1.10	1.20	1.90	2.20	4.40	1.60
Average	1.0	1.18	0.99	1.24	1.29	1.79	1.93	1.73

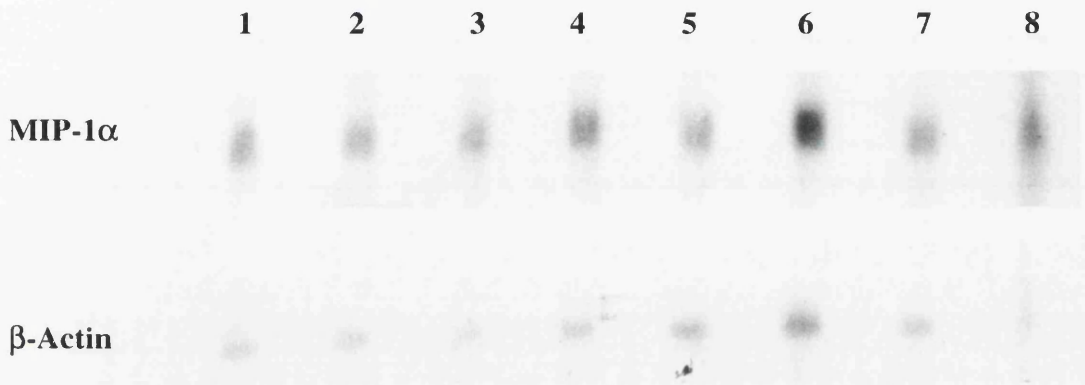


Figure 6.9: Northern blot analysis of the effect of a titration of IL-5 on MIP-1α mRNA expression in BMM.

BMM were treated for 4 h with the following concentrations of IL-5:
1) control; 2) 1 pg/ml; 3) 10 pg/ml; 4) 0.1 ng/ml; 5) 0.5 ng/ml;6) 1 ng/ml; 7) 5 ng/ml; 8) 10 ng/ml. Top panel shows a blot hybridised with a probe for MIP-1α, the bottom panel shows the same blot stripped and reprobed for β-Actin. This figure is representative of at least 3 separate experiments.

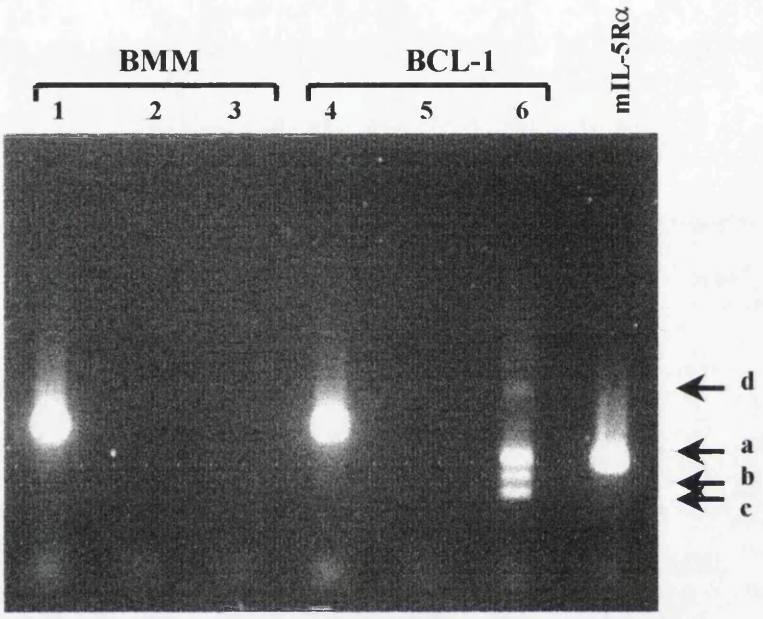


Figure 6.10: RT-PCR analysis of the expression of the IL-5Rα chain in BMM.

Total RNA was isolated and analysed for the expression of either GAPDH or IL-5Rα chain as follows: Lanes 1-3 show reactions performed using BMM RNA, whilst lanes 4-6 show reactions performed using BCL-1 RNA (a positive control cell line for mIL-5Rα chain expression). Reactions were performed using either GAPDH primers (lanes 1-3) or with primers specific for mIL-5Rα chain (lanes 4-7). Lanes 2 and 5 are negative control reactions performed without the addition of reverse transcriptase. The reaction shown in lane 7 is a positive control, performed using mIL-5Rα chain cDNA. The IL-5Rα chain primers used for the PCR reaction define fragments of the following size: arrow a) 548 bp; arrow b) 452 bp; arrow c) 369; arrow d) a possible alternatively spliced variant. This figure represents data from a single experiment.

Also included was a positive control cell line, BCL-1 (lane 6), previously shown to express the IL-5Rα chain. Four bands were observed in this and they corresponded exactly to those previous identified by Takaki *et al* (1990) as follows. Arrow a) membrane bound form; arrows b) and c) soluble IL-5R; arrow d) probably corresponds to an alternatively spliced mRNA variant. Also shown is an internal control for the housekeeping gene GAPDH (lanes 1 and 4). This data strongly suggests that BMM are unable to respond to IL-5, due to the absence of IL-5Rα chain and therefore a functional IL-5 receptor. It does not however preclude the possibility of IL-5 regulating the expression of MIP-1α in other cell types, that do express functional IL-5 receptors.

6.5.3 IL-5 does not induce expression of MIP-1α mRNA in bone marrow cells

The bone marrow consists of a heterogeneous population of cells which includes cells other than macrophages, some of which are known to be IL-5 responsive. As MIP-1α expression was undetectable in unfractionated murine bone marrow cells by Northern blot analysis, the expression of MIP-1α mRNA was examined by RT-PCR analysis in order to determine whether cells within the bone marrow could alter their expression of MIP-1α in response to IL-5. Figure 6.11 shows that MIP-1α is readily detectable in untreated bone marrow cells, but that this level of expression does not appear to be significantly altered following treatment with IL-5. This suggests that cells within the bone marrow do not readily increase their expression of MIP-1α mRNA, in response to IL-5. It is possible however, that cells may respond but that the number of responsive cells is too low to result in a significant change in the overall level of MIP-1α mRNA expression.

6.6 Effect of GM-CSF on the Expression of mRNA for other CC chemokines

MIP-1α is a member of a large superfamily of cytokines known as the chemokines which are divided into several subfamilies, with MIP-1α being a member of the CC subfamily (see introduction). It had been previously demonstrated that IL-8, a member of the CXC chemokine subfamily, was inducible in monocytes by GM-CSF and IL-3 stimulation (Takahashi *et al* 1993). Therefore, the effect of GM-CSF and

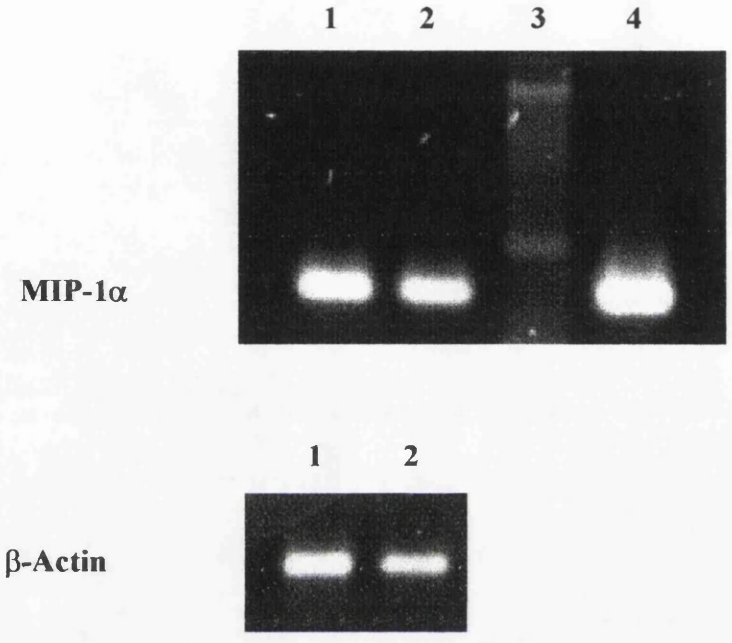


Figure 6.11: RT-PCR analysis of the effect of IL-5 on the expression of MIP-1α mRNA in murine bone marrow cells.

Bone marrow cells harvested from murine femora were treated for 4 h as follows: Lane 1) Control treated cells; lane 2) IL-5 treated (10 ng/ml). RT-PCR analysis was then used to examine the expression of MIP-1α. The reactions shown in lanes 3 and 4 are positive PCR controls performed using genomic DNA and MIP-1a cDNA, respectively. Top panel shows products of reactions performed with MIP-1α primers, whilst bottom panel shows those performed with β-Actin primers. This figure represents data from a single experiment.

IL-3 on the expression of other murine members of the CC subfamily was investigated in BMM, to examine if GM-CSF induction was specific to MIP-1α.

6.6.1 GM-CSF induces expression of MIP-1β mRNA

MIP-1β is very similar to MIP-1α, both in its expression and in many of its functions. BMM were therefore treated with either GM-CSF or IL-3 (both at 10 ng/ml) for 4 h and then the level of murine MIP-1β mRNA analysed by Northern blot analysis, using a MIP-1β-specific probe. Figure 6.12 shows that MIP-1β mRNA is induced by GM-CSF and IL-3 and densitometric analysis revealed this induction to be approximately 15 to 20-fold, a similar magnitude to that observed for MIP-1α.

6.6.2 GM-CSF and IL-3 induce expression of JE and MARC mRNA

MARC and JE are two other murine CC chemokines known to be expressed by monocytes and macrophages (see introduction). The expression of JE and MARC mRNA was examined using an identical protocol to that used for MIP-1β expression. Figures 6.13 A and 6.13 B show that both JE and MARC mRNA are substantially induced by both GM-CSF and IL-3.

These results suggest that expression of at least 3 other chemokines, in addition to MIP-1α, can be regulated by GM-CSF and implies that additional chemokines not investigated here may also be regulated in a similar fashion.

6.7. Effect of TGF-β1 on the GM-CSF and IL-3 Induced Expression of CC Chemokines

Previous work in our laboratory has documented the existence of an endogenous reciprocal relationship in BMM, between MIP-1α and TGF-β1 (Maltman *et al* 1993, 1996). It is proposed that TGF-β1 may function to suppress the expression of MIP-1α in the bone marrow, and yet MIP-1α expression is detectable (Cluitmans *et al* 1995), suggesting that other endogenous factors may act to oppose this block. In the light of the observations documented here, that GM-CSF and IL-3 are both strong inducers of MIP-1α expression in BMM, the potential relationship between TGF-β1, GM-CSF, IL-3 and MIP-1α expression was investigated.

Figure 6.12:

The numbers in the table below show the relative levels of MIP-1β mRNA for these 2 experiments, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.
* = data shown in the figure opposite

	Control	GM-CSF	Control	IL-3
	*1.00	*14.7	*1.00	*18.0
	1.00	14.5	1.00	22.0
Average	1.00	14.6	1.00	20.0

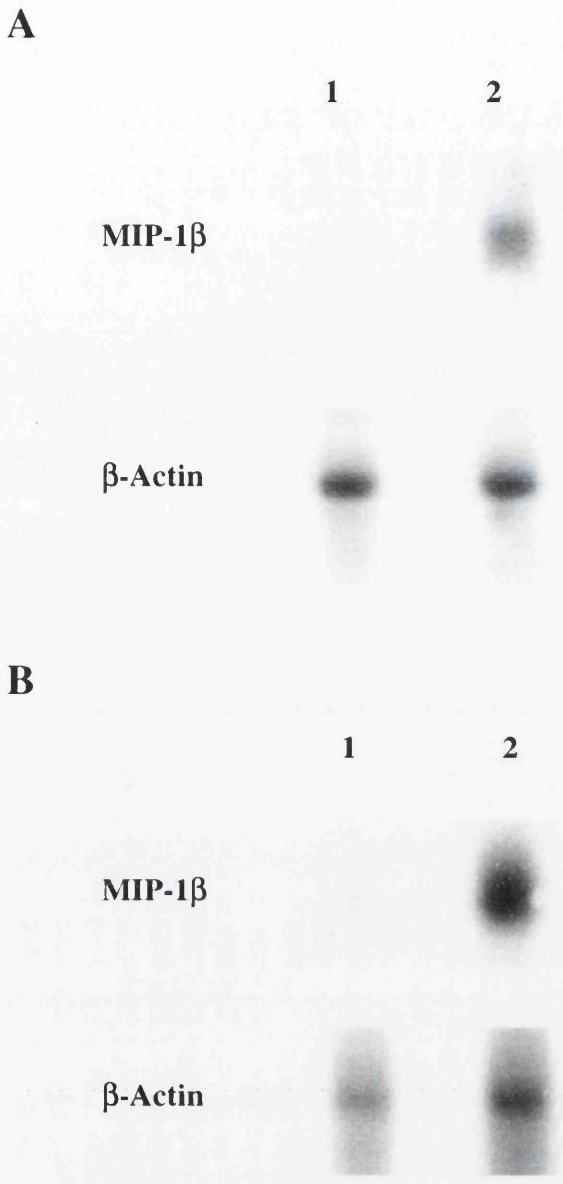


Figure 6.12: Northern blot analysis of the effect of GM-CSF and IL-3 on the expression of MIP-1β mRNA in BMM.

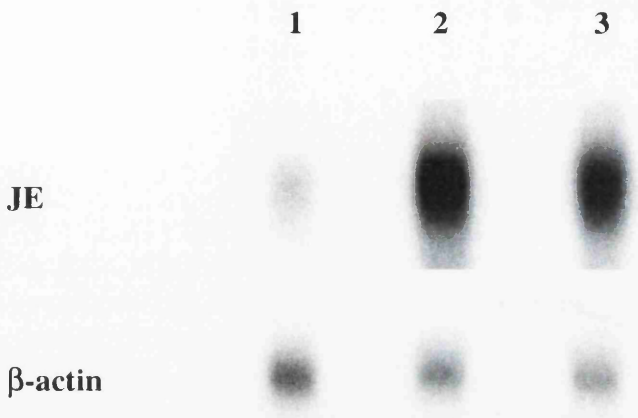
A) Effect of GM-CSF on MIP-1β expression. BMM were treated for 4 h as follows: 1) control; 2) GM-CSF (10 ng/ml). *B)* Effect of IL-3 on MIP-1β expression. BMM were treated as follows: 1) control; 2) IL-3 (10 ng/ml). The top blot of each panel shows a blot hybridised with a probe for MIP-1β, the bottom blot of each panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of 2 separate experiments.

Figure 6.13: Northern blot analysis of the effect of GM-CSF and IL-3 on the expression of JE and MARC mRNA in BMM.

A) Effect of GM-CSF and IL-3 on JE expression. BMM were treated as follows: 1) control; 2) 4 h GM-CSF (10 ng/ml); 3) 4 h IL-3 (10 ng/ml). *B)* Effect of GM-CSF and IL-3 on MARC expression. BMM were treated as in *A* above. Top blot of each panel shows a blot hybridised with a probe for either JE or MARC, the bottom blot in each panel shows the same blot stripped and reprobed for b-Actin. This figure is representative of 3 separate experiments. The table below shows the relative levels of mRNA for JE and MARC for 3 separate experiments (*= data for those experiments shown opposite in panels A and B, respectively), as assessed by scanning densitometric analysis and normalisation against the calculated level of β-Actin mRNA.

		Control	GM-CSF	IL-3
JE		*1.00	*33.0	*52.0
		1.00	25.0	62.0
		1.00	59.0	33.0
	Average	1.00	39.0	49.0
MARC				
		1.00	9.78	10.4
		1.00	10.4	7.00
		*1.00	*16.0	*25.0
	Average	1.00	12.1	14.1

A



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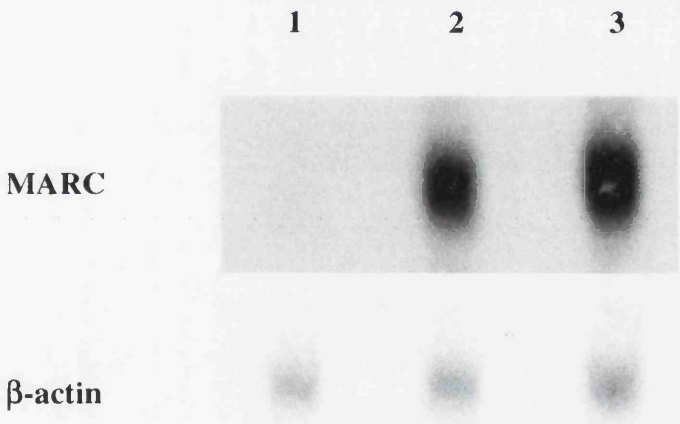


6.7.1 TGF-β1 can suppress the induction of MIP-1α mRNA expression induced by GM-CSF and IL-3

The ability of TGF-β1 to suppress MIP-1α expression in the presence of GM-CSF or IL-3 was assessed. BMM were stimulated with GM-CSF or IL-3 as described above, either in the presence or absence of TGF-β1 (20 ng/ml). A previous study in our laboratory had shown suppression of MIP-1α expression, with around 1 pM TGF-β1 (Maltman *et al* 1993). A dose of 20 ng/ml was selected for the experiments reported herein, as this was in excess of the doses used in the previous studies and therefore likely to result in suppression of MIP-1α expression, despite the presence the strong inducing stimulus of GM-CSF. Figure 6.14 shows one such experiment. As previously, densitometric analysis revealed that GM-CSF and IL-3 potently induced MIP-1α mRNA expression, approximately 20-fold, relative to control values (compare lane 1 with lanes 2 and 4). However, in the presence of TGF-β1, this induction was reduced by approximately 55-85 % (compare lane 2 with 3, and lane 4 with 5). As previously reported, TGF-β1 alone was able to suppress the level of MIP-1α expression below the basal level normally seen in unstimulated cells (compare lane 1 with 6). These data indicate that TGF-β1 is indeed able to suppress the induction of MIP-1α mRNA expression induced by GM-CSF and IL-3. However, this suppression does not appear complete despite the very high level of TGF-β1 used, suggesting that GM-CSF and IL-3 are indeed strong inducers of MIP-1α expression and may therefore be able to overcome TGF-β1 suppression of MIP-1α expression *in vivo*. These results indicate that the opposing regulatory stimuli exerted by GM-CSF and TGF-β on MIP-1α expression are probably not exclusive of each other and are also unlikely to be an "all or nothing effect". The interactions occuring *in vivo* are therefore likely to be dynamic and dependent upon the relative levels of GM-CSF, IL-3 and TGF-β present within the bone marrow microenvironment at any given time.

6.7.2 The suppression of GM-CSF induced MIP-1α expression by TGF-β1 is dose-dependent

In order to further examine the relationship between TGF-β1, GM-CSF and MIP-1α, BMM were treated with GM-CSF in the presence of a titration of TGF-β1. Figure 6.15 shows that as above, TGF-β1 suppressed the induction of MIP-1α mRNA

Figure 6.14:

The numbers in the table below show the relative levels of MIP-1α mRNA for this experiment, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells. * = data shown in the figure opposite.

	Control	GM-CSF	GM-CSF + TGF-β1	IL-3	IL-3 + TGF-β1	TGF-β1
	*1.0	*21.0	*3.30	*22.8	*10.4	N/M
	1.0	6.0	1.90	7.40	3.22	N/M
Average	1.0	13.5	2.60	15.1	6.81	N/M

N/M = not measurable

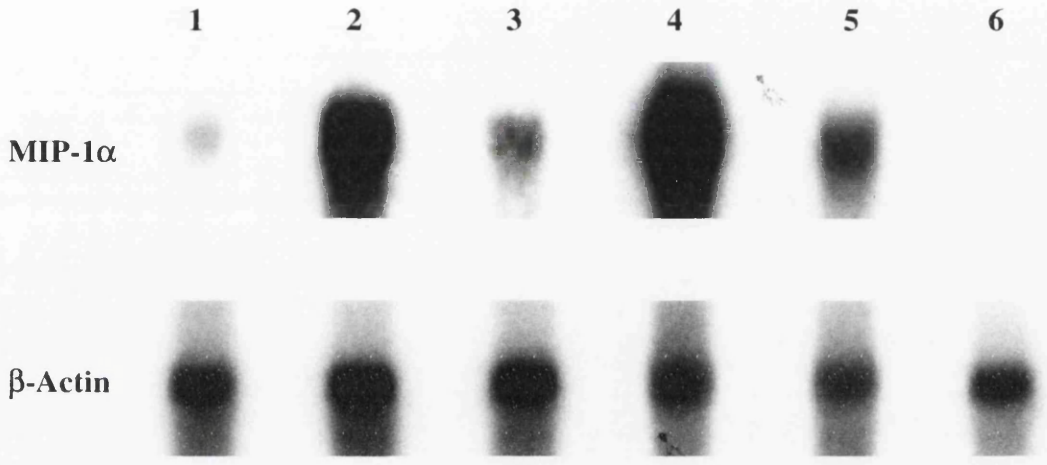


Figure 6.14: Northern blot analysis of the effect of TGF-β1 on the induction of MIP-1α mRNA expression by GM-CSF and IL-3.

BMM were stimulated for 4 h with the following additions: 1) control; 2) GM-CSF (10 ng/ml); 3) GM-CSF plus TGF-β1 (20 ng/ml); 4) IL-3 (10 ng/ml); 5) IL-3 plus TGF-β1; 6) TGF-β1. Top panel shows a blot hybridised with a probe for MIP-1α, whilst the bottom panel shows the same blot stripped and reprobed for β-Actin. This figure is representative of 2 separate experiments.

Figure 6.15:

This figure represents data from a single experiment. The numbers in the table below show the relative levels of MIP-1α mRNA for this experiment, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.

Control	GM-CSF	GM + 20ng/ml TGF-β1	GM +2ng/ml TGF-β1	GM+200pg /ml TGF-β1	GM+20pg/ ml TGF-β1
1.00	11.9	2.49	3.70	10.8	5.70

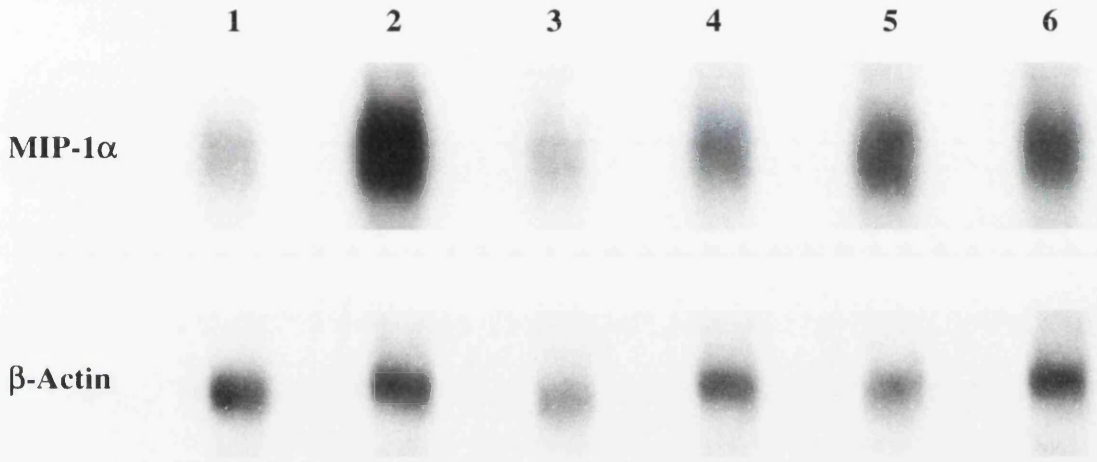


Figure 6.15: Northern blot analysis of the effect of a titration of TGF-β1 on the induction of MIP-1α mRNA expression by GM-CSF

BMM were stimulated for 4 h with the following additions: 1) control; 2) GM-CSF (10 ng/ml); 3) GM-CSF plus TGF-β1 (20 ng/ml); 4); GM-CSF plus TGF-β1 (2 ng/ml); 5) GM-CSF plus TGF-β1(200 pg/ml); 6) GM-CSF plus TGF-β (20 pg/ml). Top panel shows a blot probed with MIP-1α, whilst the lower panel shows the same blot stripped and reprobed with a probe for β-Actin.

by GM-CSF, by approximately 80 % (compare lane 1 with lanes 2 and 3). As the amount of TGF-β1 was decreased, so the level of MIP-1α expression rose (compare lanes 4, 5, and 6 with lane 3). The level of expression observed with the lowest dose of TGF-β1 (0.02 ng/ml) was unexpectedly lower than that seen with a 10-fold higher dose of TGF-β1 (lanes 6 and 5 respectively). The reason for this is unclear, but may be the result of a slightly unequal loading of RNA between lanes as indicated by the β-Actin blot. These results confirm the observations made in the previous experiment, that TGF-β1 is a suppressor of MIP-1α expression, but that GM-CSF may be sufficiently potent to at least partially overcome TGF-β1 mediated suppression.

6.7.3 TGF-β1 can also suppress the induction of JE and MARC mRNA expression induced by GM-CSF and IL-3

As described in section 6.6.2, GM-CSF and IL-3 can induce the expression of several chemokines in addition to that of MIP-1α. This observation suggested that TGF-β might have the potential to suppress the induction of these additional chemokines. The expression of MARC and JE mRNA following GM-CSF or IL-3 treatment was therefore examined in the presence of TGF-β1 (20 ng/ml). Figure 6.16 shows that like MIP-1α, the induction of both MARC and JE by GM-CSF or IL-3, can be suppressed in the presence of TGF-β1 (by approximately 50-75 %). As before, this TGF-β1 suppression was not absolute and some induction of both MARC and JE expression was still evident in the presence of GM-CSF or IL-3, relative to control values. As with MIP-1α, in the absence of either GM-CSF or IL-3, the basal levels of both MARC and JE appeared to be suppressed by TGF-β. These results indicate that, as with MIP-1α, the basal or the induced expression of other CC chemokines can be negatively regulated by TGF-β1.

6.8 Effect of GM-CSF, IL-3 and TGF-β1 on the Expression of MIP-1α Receptors

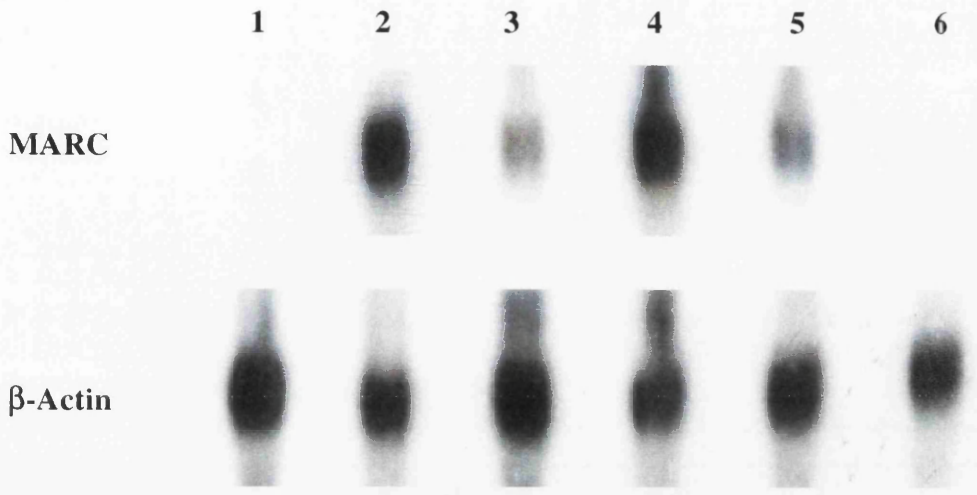
As outlined in the introduction, the last few years have seen a rapid growth in the understanding of the interactions between chemokines and their cognate receptors. Many chemokines, including MIP-1α, are somewhat unusual in their ability to bind and functionally activate more than one receptor. In fact, as indicated in table 3.1, MIP-1α has been reported to be a functional ligand for at least 4 receptors. To date,

Figure 6.16: Northern blot analysis of the effect of TGF-β1 on the induction of MARC and JE mRNA expression by GM-CSF and IL-3.

A) Effect of TGF-β1 on the induction of MARC mRNA by GM-CSF and IL-3. BMM were treated for 4 h under the following conditions 1) control; 2) GM-CSF (10 ng/ml); 3) GM-CSF plus TGF-β1 (20 ng/ml); 4) IL-3 (10 ng/ml); 5) IL-3 plus TGF-β1; 6) TGF-β1. *B)* Effect of TGF-β1 on the induction of JE mRNA by GM-CSF and IL-3. BMM were treated as *A)* above. Top blot in each panel shows a blot hybridised with a probe for either MARC or JE, whilst the bottom blot in each panel shows the same blot stripped and reprobed for β-Actin. This figure is representative of 2 separate experiments. The table below shows the relative levels of mRNA for MARC and JE for these 2 separate experiments (*= data for those experiments shown opposite in panels A and B, respectively), as assessed by scanning densitometric analysis and normalisation against the calculated level of β-Actin mRNA.

	Control	GM-CSF	GM-CSF/TGF-β	IL-3	IL-3/TGF-β	TGF-β
JE	1.00	122	70.3	121	51.4	N/M
	*1.00	*62.3	*31.4	*92.0	*25.0	N/M
Average	1.00	92.1	50.9	49.0	38.2	N/M
MARC	*1.00	*119	*48.6	*120	*65	N/M
	1.00	210	69.3	325	143	N/M
Average	1.00	165	58	223	104	N/M

A



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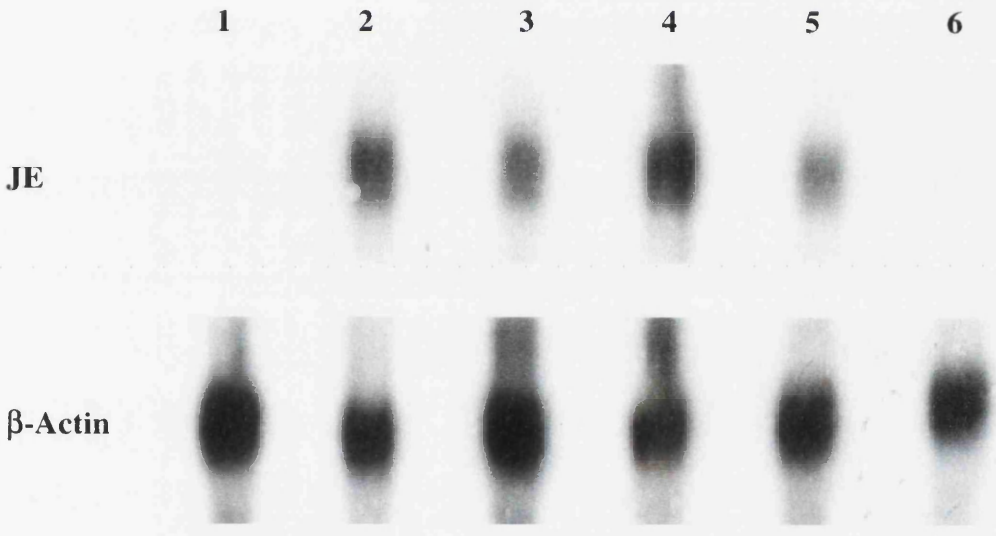


whilst some progress has been made in understanding the regulation of MIP-1α expression, very little is known regarding the regulatory controls governing the expression of the various MIP-1α receptors. Given the potent inductive effect of GM-CSF and IL-3 on MIP-1α expression reported in the previous sections, it was decided to examine whether GM-CSF and IL-3 could also affect the expression of MIP-1α receptors.

6.8.1 GM-CSF and IL-3 increase the expression of CCR1 in BMM, but not that of CCR5

As discussed in the introduction, the CC chemokine receptors CCR1 and CCR5 are known to be widely expressed on cells of the macrophage lineage. Therefore the expression of CCR1 and CCR5, together with that of CCR3, CCR4 and D6, was examined in BMM using northern blot analysis. No expression of CCR3, CCR4, and D6 was visible in BMM, as assessed by northern blot analysis. Densitometric analysis revealed that the expression of CCR1 mRNA was increased approximately 3-5 fold following GM-CSF or IL-3 stimulation of BMM, relative to control values (see figure 6.17 panel A, compare lanes 2 and 3 with lane 1). This was the typical level of expression observed following GM-CSF stimulation, although as much as a 10-fold increase in expression was observed in some experiments. In contrast however, no change in the level of CCR5 expression was observed following treatment with GM-CSF or IL-3 (see figure 6.17 panel B, compare lanes 2 and 3 with lane 1).

6.8.2 GM-CSF and IL-3 also regulate the expression of CCR1, but not that of CCR5, in splenic cells

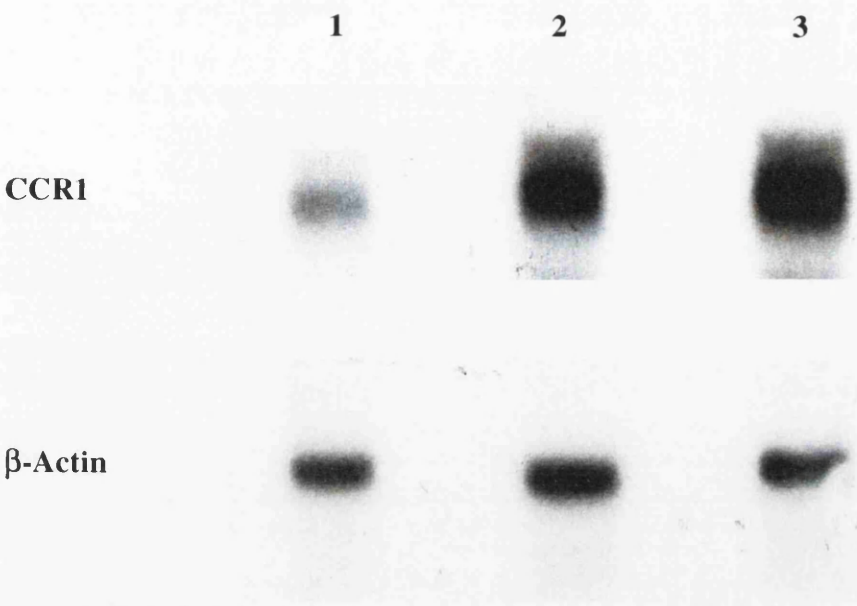
Splenic cells were also used, in an crude attempt to examine the expression of CCR1 and CCR5 in other cell types. Figure 6.18 shows that a very similar pattern of expression for CCR1 and CCR5 is evident in splenic cells, as is observed in BMM. As in BMM, CCR1 expression is elevated following stimulation of splenic cells with GM-CSF or IL-3, but CCR5 expression remains unaltered. The normal murine spleen is comprised mainly of a mixture of B and T cells and erythrocytes. Given the few numbers of macrophages present in normal mouse spleen, it is highly unlikely that the observed increase in CCR1 expression observed by Northern blotting, was

Figure 6.17: Northern blot analysis of the effect of GM-CSF and IL-3 on the expression of CCR1 and CCR5 mRNA in BMM.

A) Effect of GM-CSF and IL-3 on CCR1 expression. 1) control; 2) 4 h GM-CSF (10 ng/ml); 3) 4 h IL-3 (10 ng/ml). *B)* Effect of GM-CSF and IL-3 on CCR5 expression. BMM were treated as *A)* above. Top blot of each panel shows a blot hybridised with a probe for either CCR1 or CCR5, whilst the bottom blot in each panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of 3 separate experiments. The table below shows the relative levels of mRNA for CCR1 and CCR5 for these 3 separate experiments (*= data for those experiments shown opposite in panels A and B, respectively), as assessed by scanning densitometric analysis and normalisation against the calculated level of β-Actin mRNA.

		Control	GM-CSF	IL-3
CCR1		*1.00	*3.10	*4.60
		1.00	4.76	6.80
		1.00	9.06	8.96
	Average	1.00	5.64	6.79
CCR5				
		*1.00	*1.00	*0.85
		1.00	1.16	0.91
		1.00	1.43	1.24
	Average	1.00	1.20	1.00

A



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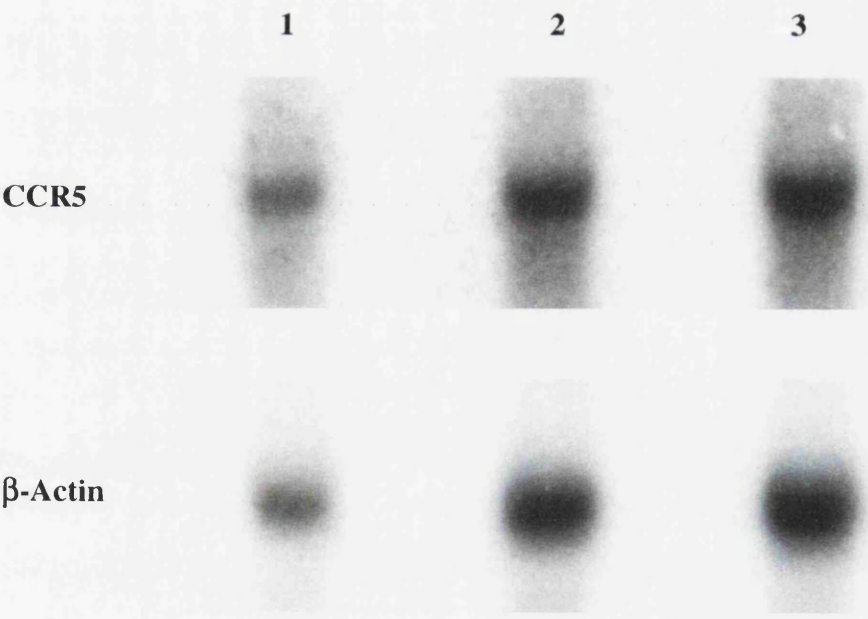
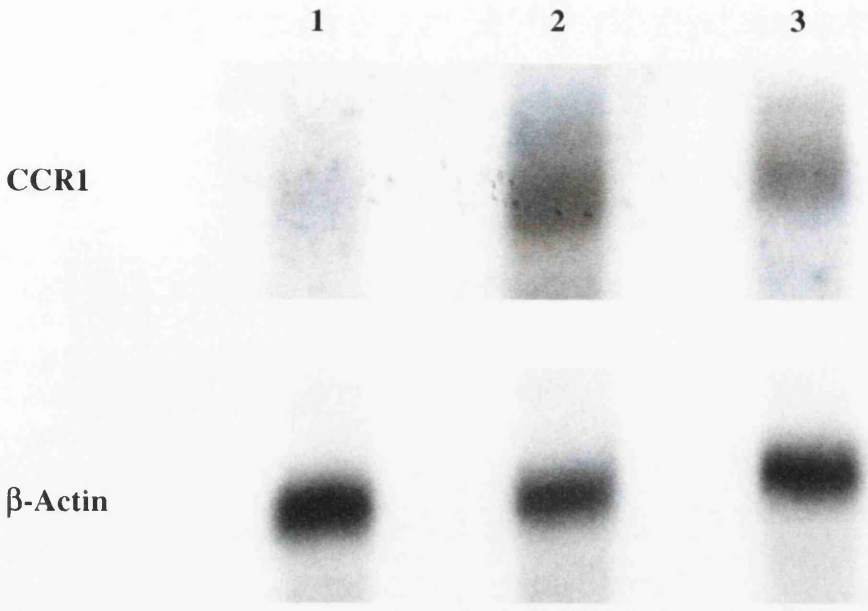


Figure 6.18: Northern blot analysis of the effect of GM-CSF and IL-3 on the expression of CCR1 and CCR5 mRNA in splenic cells.

A) Effect of GM-CSF and IL-3 on CCR1 expression. Splenic cells were treated as follows 1) control; 2) 4 h GM-CSF (10 ng/ml); 3) 4 h IL-3 (10 ng/ml). *B)* Effect of GM-CSF and IL-3 on CCR5 expression. Splenic cells were treated as *A)* above. Top blot of each panel shows a blot hybridised with a probe for either CCR1 or CCR5, whilst the bottom blot in each panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of 2 separate experiments. The table below shows the relative levels of mRNA for CCR1 and CCR5 for these 2 separate experiments (*= data for those experiments shown opposite in panels A and B, respectively), as assessed by scanning densitometric analysis and normalisation against the calculated level of β-Actin mRNA.

		Control	GM-CSF	IL-3
CCR1		*1.00	*4.98	*3.20
		1.00	2.47	1.49
	Average	1.00	3.73	2.36
CCR5				
		*1.00	*1.44	*1.43
		1.00	1.82	0.93
	Average	1.00	1.63	1.18

A



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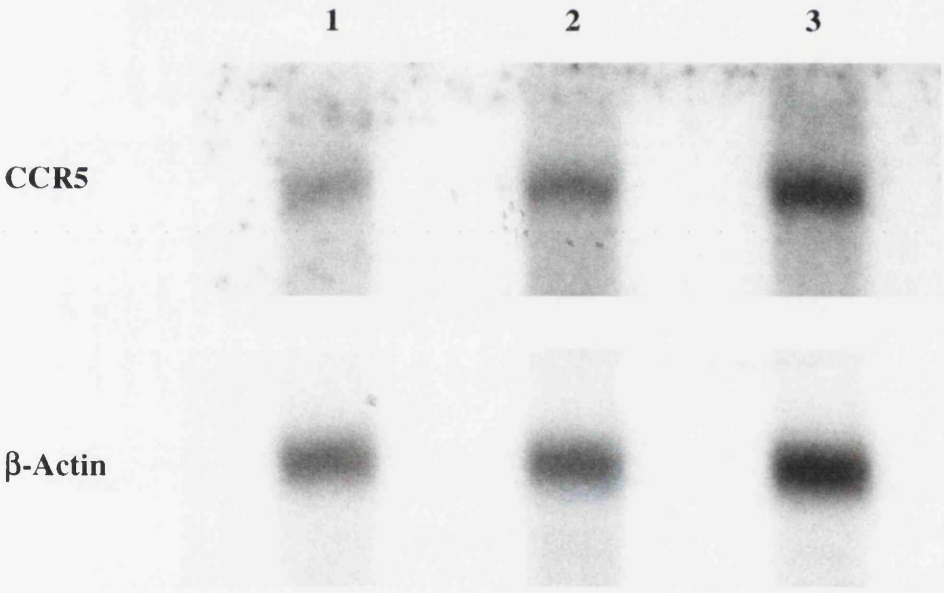


macrophage derived. This data therefore suggests that increased CCR1 expression may also occur outwith the bone marrow in non-macrophage cell types, in response to GM-CSF and IL-3.

6.8.3 GM-CSF and IL-3 do not affect the expression of other MIP-1α receptors in BMM

As indicated above, northern blot analysis of BMM failed to reveal detectable expression of mRNA for other MIP-1α receptors, namely CCR4 and D6. Nor was expression of murine CCR3 detected, a receptor which may, or may not, be a functional MIP-1α receptor and which was previously reported to be expressed in monocytes (Graham *et al* 1996) (data not shown). The highly sensitive technique of RT-PCR was therefore used to examine the expression of these receptors in BMM and splenic cells. Panel A of figure 6.19 shows that no expression of either CCR3 or CCR4 was detected in BMM, either in the presence, or absence of GM-CSF or IL-3. Included were positive controls for CCR3 (lane 8) and CCR4 (lane 12), as well as those for the housekeeping gene GAPDH (lanes 1-4), to show that the RT-PCR had indeed worked successfully and allow some inferences to be made concerning the loading between lanes. A similar observation was made in splenic cells (data not shown).

Panel B of figure 6.19 shows that expression of CCR2, which does not bind MIP-1α, was detectable in BMM but its expression was not altered by the addition of GM-CSF (compare lane 4 with lane 5). As before, a positive control for CCR2 (lane 6) and internal controls for β-Actin (lanes 1-3) were included.

Panel C of figure 6.19 shows that expression of D6 was also undetectable in BMM, either in the presence or absence of GM-CSF (compare lane 4 with lane 5). Also included was a positive control for D6 (lane 6) and internal controls for β-Actin (lanes 1-3).

6.8.4 TGF-β1 does not markedly alter the expression of CCR1 and CCR5 in BMM

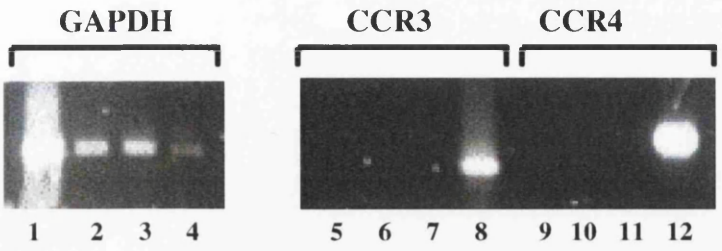
As outlined in previous sections, TGF-β1 appears to be a potent suppressor of GM-CSF and IL-3 induced MIP-1α expression. With the knowledge that GM-CSF

Figure 6.19: RT-PCR analysis of the expression of CCR3, CCR4, D6 and CCR2 following stimulation with GM-CSF or IL-3.

BMM were treated for 4 h with GM-CSF and IL-3, total RNA isolated and RT-PCR analysis performed to determine the expression of various CC chemokine receptors as follows:
A) CCR3 and CCR4 expression. Lanes 1, 5 and 9 show reactions performed with control RNA; lanes 2, 6 and 10 show reactions performed with RNA derived from cells treated for 4 h with GM-CSF (10 ng/ml); lanes 3, 7 and 11 show reactions performed with RNA derived from cells treated for 4 h with IL-3 (10 ng/ml); lanes 4, 8 and 12 show positive control reactions performed with cDNAs for GAPDH, CCR3, and CCR4 respectively. Lanes 1-4 show reactions performed with GAPDH primers, whilst lanes 5-8 and 9-12 show reactions performed with primers for CCR3 and CCR4 respectively.
B) CCR2 expression. Lanes 1 and 4 show reactions performed with control RNA; lanes 2 and 5 show reactions performed with RNA derived from cells treated for 4 h with GM-CSF (10 ng/ml); lanes 3 and 6 show positive control reactions performed with cDNAs for β-Actin and CCR2 respectively. Lanes 1-3 show reactions performed with primers for β-Actin, whilst lanes 4-6 show reactions performed with primers for CCR2.
C) D6 expression. Lane assignments as for *B)* above, except that reactions in lanes 4-6 were performed with primers for D6.

This figure is representative of 3 separate experiments.

A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CCR3
CCR4
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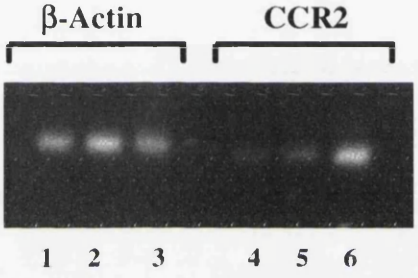
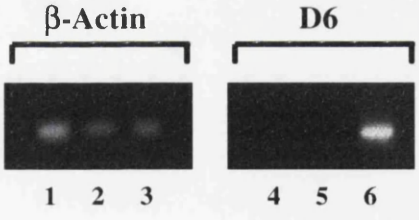
5 6 7 8 9 10 11 12

B


β-Actin
CCR2
1 2 3 4 5 6

C


β-Actin
D6
1 2 3 4 5 6

and IL-3 also induce the expression of CCR1, but not CCR5, the effect of TGF-β1 on CCR1 and CCR5 expression was also examined. Figure 6.20 shows that whilst TGF-β1 does decrease the basal expression of both CCR1 and CCR5 following addition of GM-CSF or IL-3, the expression of CCR1 was induced as previously in the presence of GM-CSF (compare lanes 2 and 3, and lanes 4 and 5, with that of lane 1). This basal suppression of approximately 10-30 % was minimal in comparison to the 50-80 % suppression observed previously. TGF-β1 appears to suppress only the basal level of expression of both CCR1 and CCR5. This basal suppression is most obvious when cells treated with TGF-β1 are compared to control cells (compare lane 6 with the negative control, lane 1), an observation also previously noted for MIP-1α. These data suggests that whilst TGF-β1 may act to suppress basal expression of CCR1 and CCR5, it does not appear to markedly alter their expression following stimulation with GM-CSF or IL-3.

6.9 Effect of GM-CSF on MIP-1α Receptor Levels on BMM

To assess whether the observed increase in CCR1 mRNA expression that occurred following GM-CSF stimulation was of functional relevance, the effect of GM-CSF on the BMM cell surface expression of MIP-1α receptors was examined. BMM were either stimulated with GM-CSF (10 ng/ml) or with PBS / 0.1 % BSA (v/v) for 16 h and then radioligand displacement studies were performed in the presence of 4 nM ^{125}I labelled MIP-1α and variable amounts of cold MIP-1α competitor. The extended GM-CSF treatment time of 16 h, compared with the 4 h used for Northern blot analysis, was to allow sufficient time for CCR1 protein expression on the cell surface to reflect the mRNA levels. One such experiment is shown in figure 6.21 and shows a Scatchard analysis of displacement of radiolabelled MIP-1α from control and GM-CSF treated BMM. This Scatchard analysis was performed using the Ligand software program, widely used for the analysis of receptor binding data. The plot indicates several things. Firstly, the binding of MIP-1α to cell surface receptors appeared to occur with relatively similar affinities, with K_d's (dissociation constant)of 0.67 nM and 0.2 nM for control and GM-CSF treated BMM respectively (as indicated by the gradients of the slopes of the lines). Secondly, the number of MIP-1α receptors increased following GM-CSF treatment, from over 20,000 to 46,000 receptors per

Figure 6.20:

The numbers in the table below show the relative levels of CCR1 and CCR5 mRNA for these 3 experiments, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells.
* = data shown in the figure opposite.

	Control	GM-CSF	GM-CSF + TGF-β1	IL-3	IL-3 + TGF-β1	TGF-β1
CCR1	*1.00	*3.10	*2.70	*4.60	*3.60	*0.09
	1.00	2.33	1.60	1.90	1.89	0.14
	1.00	4.09	4.05	3.77	1.77	0.73
Average	1.00	3.17	2.78	3.42	2.42	0.32
CCR5	*1.00	*1.43	*1.06	*1.11	*0.86	*0.47
	1.00	1.10	0.58	0.91	0.65	0.30
	1.00	1.80	0.77	1.34	0.56	0.32
Average	1.00	1.44	0.80	1.12	0.69	0.36

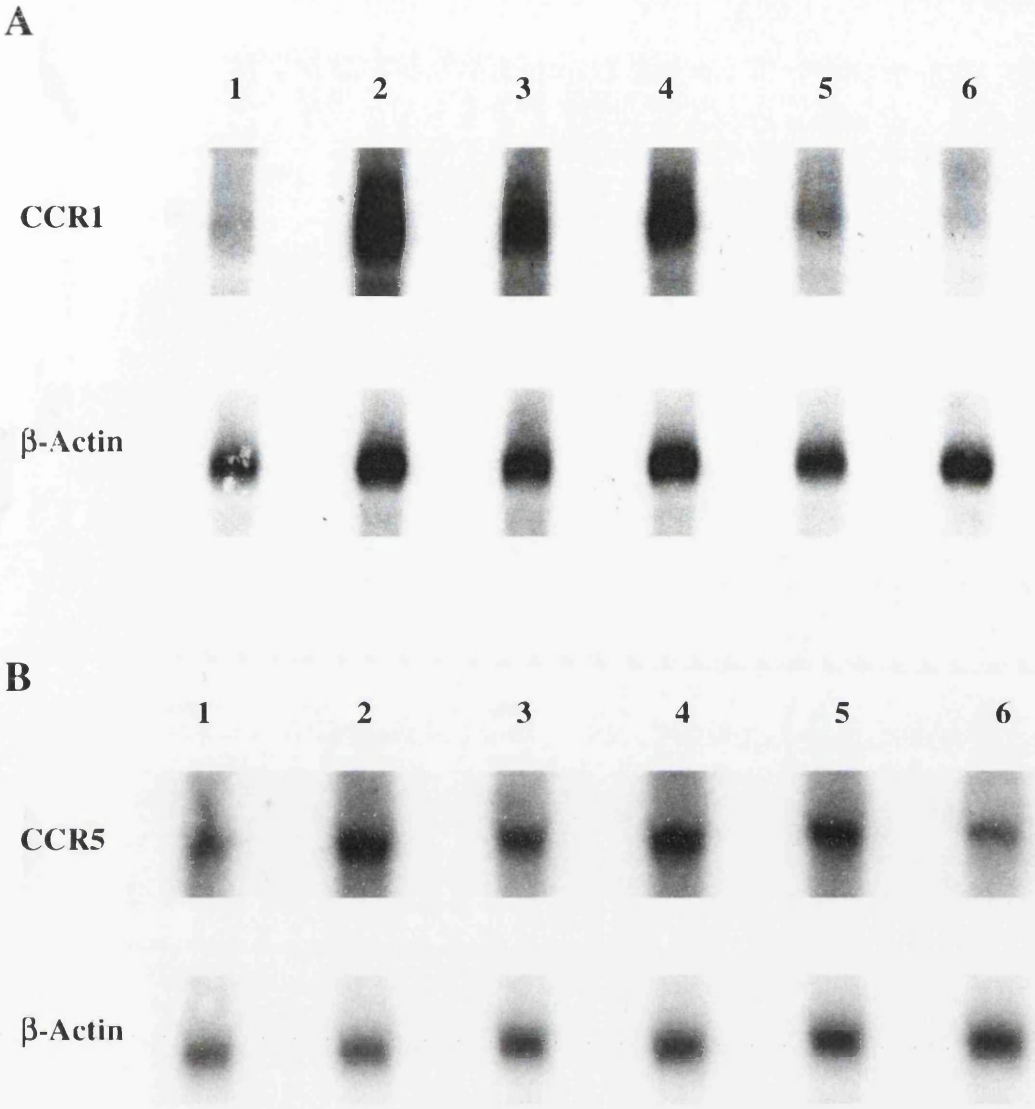


Figure 6.20: Northern blot analysis of the effect of TGF-β1 on the expression of CCR1 and CCR5 mRNA, in the presence of GM-CSF and IL-3.

BMM were treated for 4 h with GM-CSF or IL-3, in the presence or absence of TGF-β1, as follows: *A)* CCR1 expression. 1) control; 2) GM-CSF (10 ng/ml); 3) GM-CSF plus TGF-β1 (20 ng/ml); 4) IL-3 (10 ng/ml); 5) IL-3 plus TGF-β1 (20 ng/ml); 6) TGF-β1. *B)* CCR5 expression. As *A)* above. Top blot of each panel shows a blot hybridised with a probe for either CCR1 or CCR5, whilst the bottom blot in each panel shows the same blot stripped and reprobed for β-Actin. This figure is representative of 3 separate experiments.

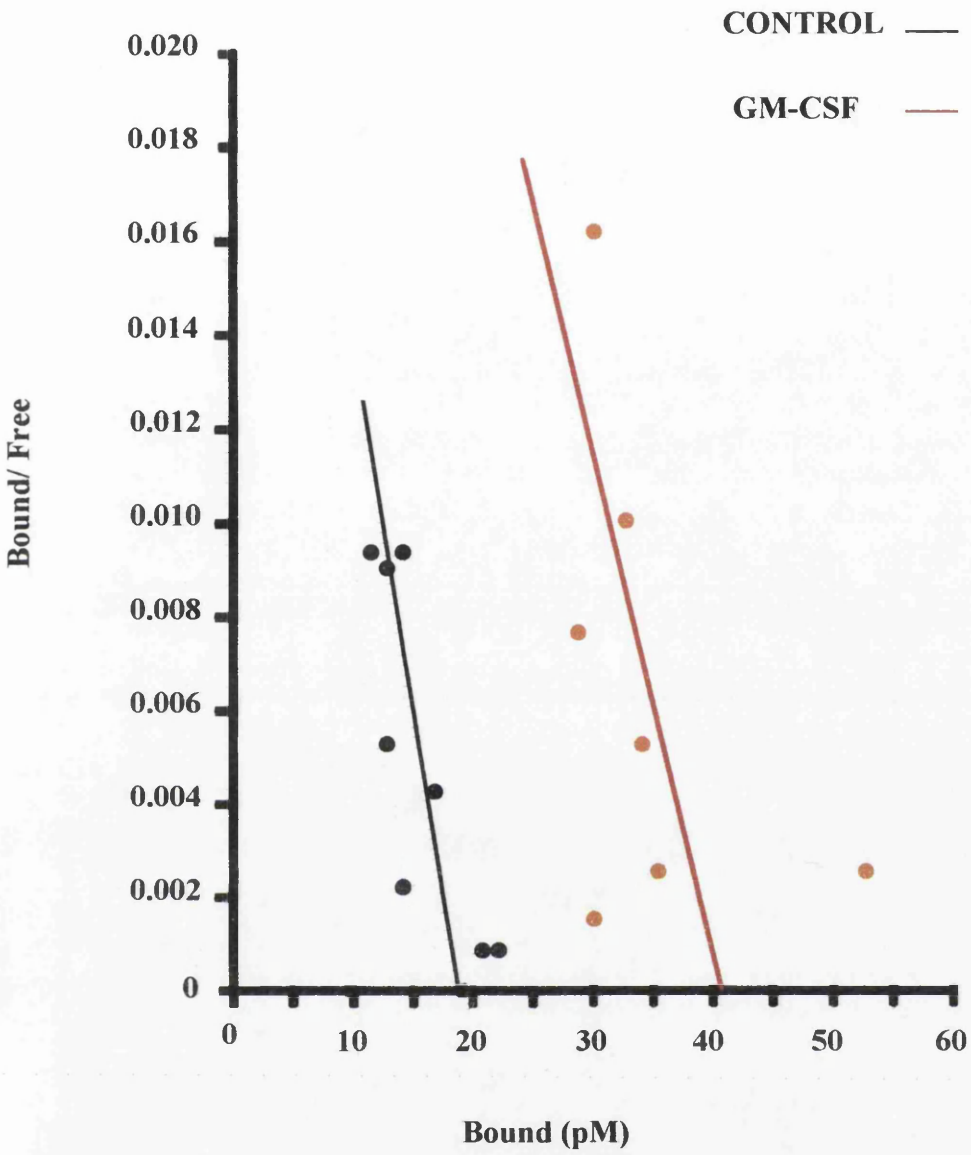


Figure 6.21 Scatchard analysis of the effect of GM-CSF on the binding of ^{125}I MIP-1α to MIP-1α receptors on BMM.

BMM were treated for 16 h with either a PBS/0.1 % BSA control or with GM-CSF (10 ng/ml). Radioligand displacement studies were then performed either with or without the addition of variable amounts of cold competitor MIP-1α. To eliminate self-aggregation, the MIP-1α used in this experiment was the non-aggregating PM2 mutant (Graham *et al* 1994). This analysis represents data derived from a single experiment.

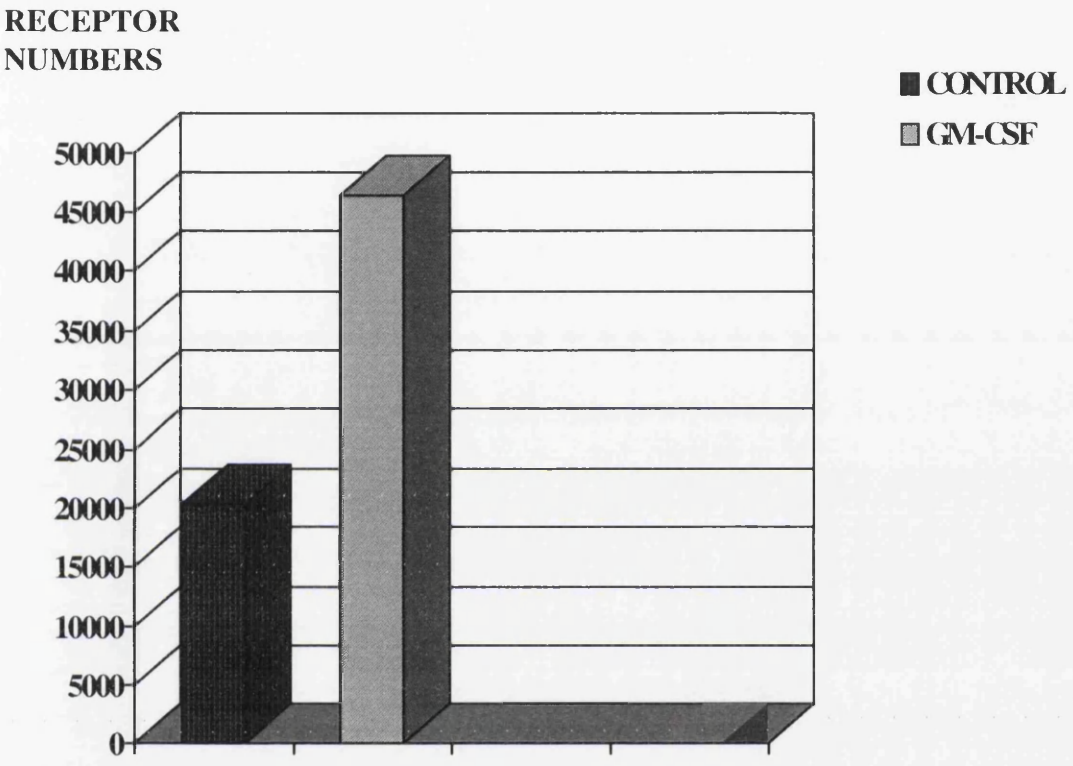


Figure 6.22 Plot of the effect of GM-CSF on BMM MIP-1α receptor numbers.

Data obtained from radiolabelled binding studies (as shown in figure 6.9.1), was analysed using the 'ligand' software package, from which the number of cell surface MIP-1α receptors was determined (as indicated on the Y axis).

cell, equating to approximately a 130 % increase (summarised in figure 6.22). It was not possible to determine exactly which MIP-α receptor was being elevated on BMM, following GM-CSF treatment, using the binding studies as performed herein. However, given the fact that BMM appear to express only CCR1 and CCR5, it is highly likely that the observed increase in MIP-1α receptor numbers could only be attributable to expression of one of these two receptors. Further support for this is provided by the similarity of K_d values observed in this study, to those previously reported for murine CCR1 (Nibbs *et al* 1996). Given the fact that the Scatchard analysis suggested only a single class of MIP-1α receptor, coupled with the previous failure to detect any increase in the expression of other known MIP-1α receptors, it is most likely that an increase in the level of CCR1 was responsible for the observed increase in number of macrophage MIP-1α cell surface receptors following GM-CSF treatment. It is also theoretically possible however, that this increase could have been due in part to an increase in an as yet unidentified MIP-1α receptor present on BMM, though the apparent existence of only a single class of MIP-1α receptor would suggest otherwise.

6.10 Effect of GM-CSF on the Ability of Cells Expressing MIP-1α Receptors to Mobilise Calcium in Response to MIP-1α

6.10.1 Effect of GM-CSF on the ability of BMM to mobilise calcium in response to MIP-1α

The data obtained from the binding studies on BMM, as indicated in the above section, suggested that treatment of BMM with GM-CSF resulted in an increase in the total number of MIP-1α receptors. This increase was in the region of 130 % and it is certainly feasible to speculate that an increase of this magnitude might be sufficient to result in an alteration in the functional response of GM-CSF stimulated BMM to MIP-1α, relative to control BMM. One widely used standard for assessing the functional interaction between a chemokine and its receptor, is the ability of the activated receptor to mobilise calcium from intracellular stores within the cytoplasm. This can be measured *in vitro*, using the technique of 'calcium fluxing'. Therefore, the effect of

a 16 h GM-CSF stimulation on the ability of BMM to mobilise calcium was measured, according to previously detailed methods (see methods).

HEK-293 cells transfected with human CCR5, were included as a positive control and to verify the functional ability of the exogenously added recombinant MIP-1α. Figure 6.23 shows one such experiment. The addition of MIP-1α (100 ng/ml), as indicated by an arrow, resulted in only a tiny flux peak in BMM and this was observed in both unstimulated and GM-CSF stimulated BMM (Panels B and C). This is more evident when the fluxes obtained with BMM were compared to those obtained with the control HEK-293 cells transfected with hCCR5 (panel A). Although the fluxes did appear to be extremely small, the results of the experiment shown in figure 6.23, suggest that GM-CSF may have a potential effect on the ability of BMM to mobilise calcium in response to MIP-1α. BMM did flux, with a peak of 6 intensity units (see figure legend 6.23) in the presence of GM-CSF, compared to a peak of 2 units observed for the control BMM. It should be noted however, that the experiment shown in figure 6.23 was the only one to show such an increase and this result was not reproducible. In fact, in most cases BMM failed to flux at all.

This data implies that whilst BMM may have the potential ability to flux calcium and that this may be enhanced by GM-CSF, this increase was small and inconclusive. BMM appear therefore, to be an inappropriate system in which to measure calcium mobilisation in response to MIP-1α.

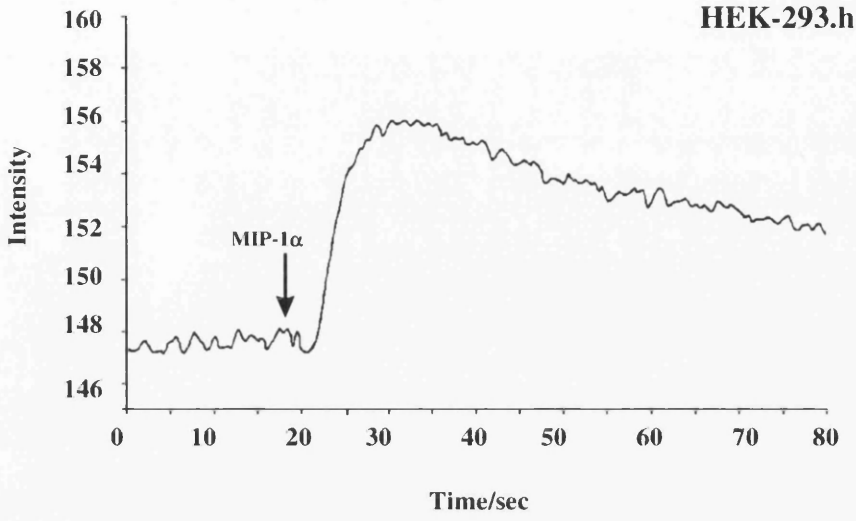
6.10.2 GM-CSF induces the expression of CCR1 mRNA in THP-1 cells

As discussed in the above section, BMM appear to be unsuitable cell choice for performing calcium flux studies in response to MIP-1α. Therefore, in order to further analyse the observed effect of GM-CSF on the ability of macrophages/monocytes to mobilise calcium in response to MIP-1α, another cell type was selected. The human pro-monocytic cell line THP-1 had been previously used with success for MIP-1α calcium flux studies in our laboratory (R.J.B. Nibbs, personal communication) and was therefore considered as a suitable alternative. The effect of a 4 h addition of GM-CSF (10 ng/ml) on the expression of CCR1 mRNA in THP-1 cells was therefore measured by Northern blot analysis and one such experiment is shown in figure 6.24. Densitometric analysis showed that treatment of

Figure 6.23 Effect of GM-CSF stimulation on the ability of BMM to mobilise calcium, in response to MIP-1α.

BMM were treated with GM-CSF (10 ng/ml) as indicated below and after this time Ca^{2+} studies were performed. *A)* shows HEK-293 cells transfected with human CCR5, as a positive control; *B)* control BMM; *C)* BMM plus 16 h GM-CSF. The point at which MIP-1α was added is indicated by an arrow. The Y axis shows the intensity of Fura-2-AM emission at 500 nm. This figure is one of 3 separate experiments.

A


HEK-293.hCCR5
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158
156
154
152
150
148
146
Intensity
MIP-1α
0
10
20
30
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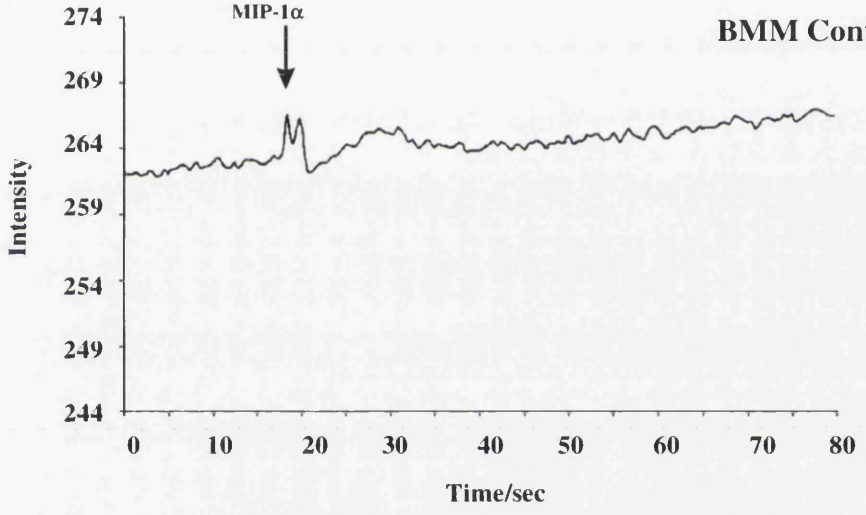
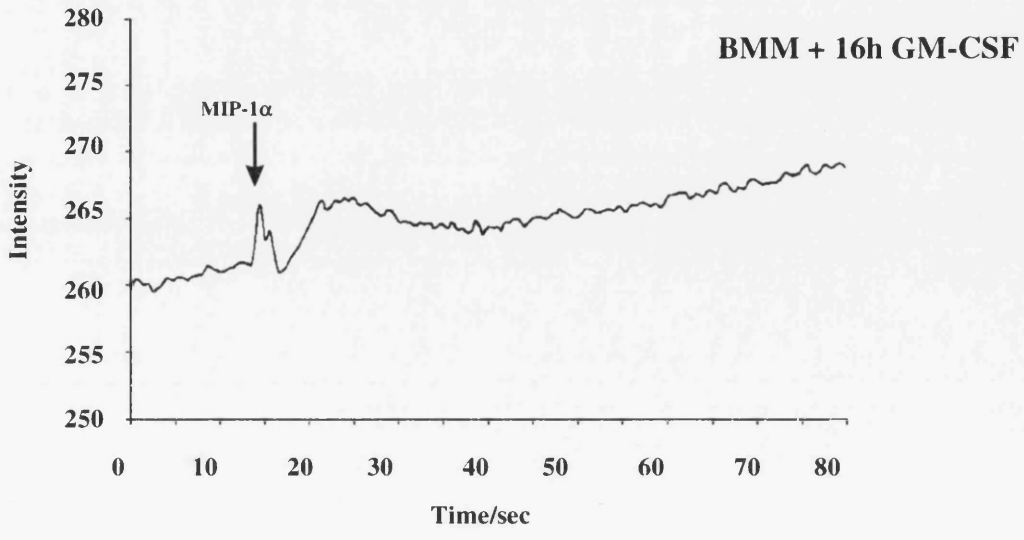
50
60
70
80
Time/sec

B


BMM Control
274
269
264
259
254
249
244
Intensity
MIP-1α
0
10
20
30
40
50
60
70
80
Time/sec

C


BMM + 16h GM-CSF
280
275
270
265
260
255
250
Intensity
MIP-1α
0
10
20
30
40
50
60
70
80
Time/sec

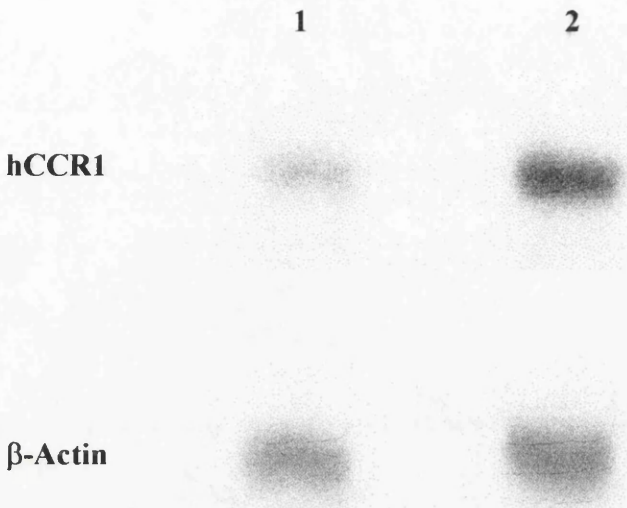


Figure 6.24: Northern blot analysis of the effect of human GM-CSF on the expression of human CCR1 mRNA in THP-1 cells.

THP-1 cells were treated as follows: 1) control 2) 4 h with human GM-CSF (10 ng/ml). The top blot shows a blot hybridised with a probe for human CCR1, the bottom panel shows the same blot, stripped and reprobed for β-Actin. This figure is representative of 3 separate experiments. The numbers in the table below show the relative levels of CCR1 mRNA for these 2 experiments, as assessed by scanning densitometric analysis and normalisation against the level of β-Actin mRNA in control treated cells. * = data shown in the figure opposite

	Control	GM-CSF
hCCR1	*1.00	*1.75
	1.00	1.90
	1.00	1.59
Average	1.00	1.75

THP-1 cells with human GM-CSF resulted in approximately a 2-fold increase in the level of CCR1 mRNA expression, which is of a slightly lower magnitude than that observed in BMM. This suggests that THP-1 cells are similar to BMM, in that they respond to GM-CSF by upregulating the expression of CCR1 and therefore may be a more suitable choice of cell, in which to examine the effect of GM-CSF on calcium mobilisation.

6.10.3 GM-CSF Does Not Alter the Ability of THP-1 cells to Mobilise Calcium in Response to MIP-1α

As previously described for BMM, the effect of a 16 h GM-CSF stimulation on the ability of THP-1 cells to mobilise calcium was measured. As before, HEK-293 cells transfected with human CCR5 were included as a positive control (see figure 6.25 panel A). Figure 6.25, shows that unlike BMM, unstimulated THP-1 cells respond well to addition of recombinant MIP-1α. However, no increase in the ability of THP-1 cells to mobilise calcium was observed following GM-CSF stimulation. GM-CSF treated THP-1 cells responded to MIP-1α with a peak flux of approximately 20 units, compared to the peak of 23 units observed with control THP-1 cells (compare panel B with panel C). This data suggests that whilst GM-CSF does increase the expression of CCR1, this increased expression does not alter the ability of those cells to mobilise calcium and it is possible therefore that the 2-fold increase in CCR1 expression is not functionally relevant. Although as discussed previously in section 6.10.1, a functional increase in BMM in response to MIP-1α still remains a theoretical possibility, as the experiments shown in figure 6.25 were performed in THP-1 cells.

6.11 Analysis of MIP-1α Expression in GM-CSF Transgenic Mouse Tissues

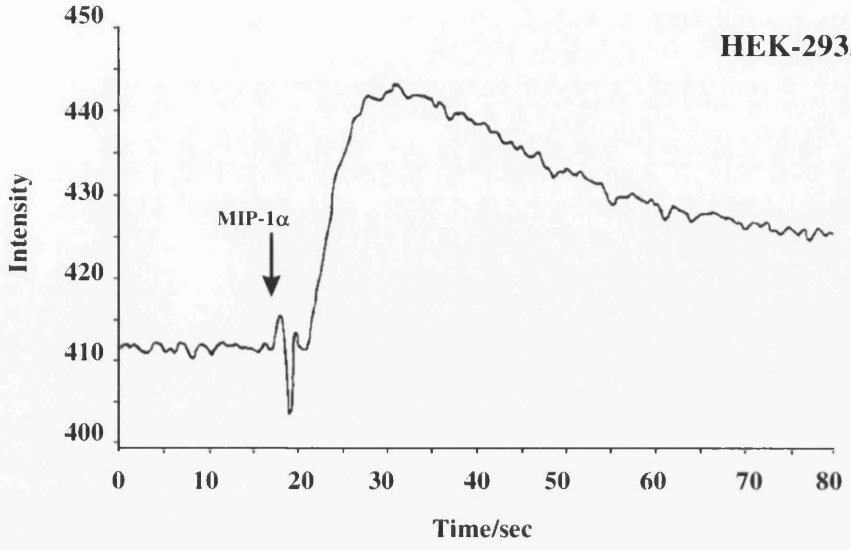
6.11.1 Overview: A potential link between MIP-1α and the pathologies observed in GM-CSF transgenic mice

So far, the results presented in this study have indicated a role for GM-CSF in the regulation of MIP-1α expression *in vitro*. Therefore, it was decided to examine the possibility that GM-CSF might be an important regulator of MIP-1α function *in vivo*. As discussed in the introduction, derivation and analysis of GM-CSF transgenic mice has previously been reported (Lang *et al* 1987). These mice are characterised by

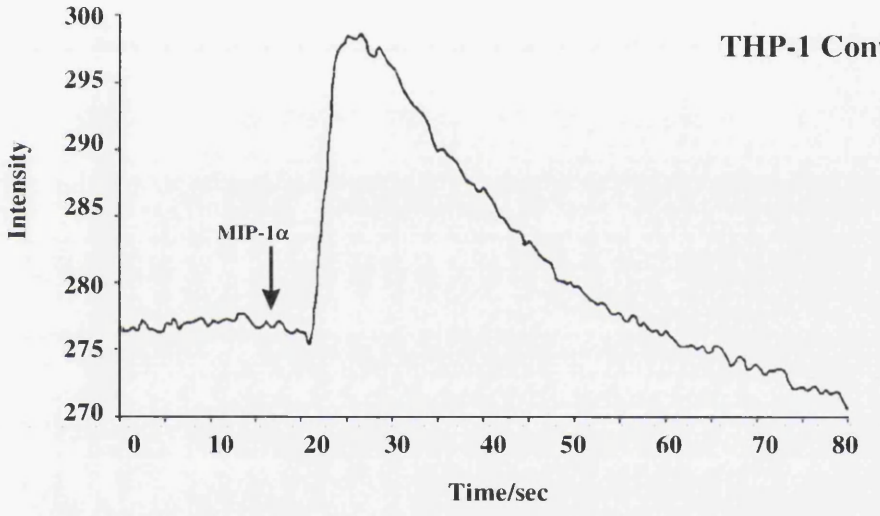
Figure 6.25 Effect of GM-CSF stimulation on the ability of THP-1 cells to mobilise calcium, in response to MIP-1α.

THP-1 cells were treated with human GM-CSF (10 ng/ml) as indicated below and after this time Ca^{2+} studies were performed. *A)* shows HEK-293 cells transfected with human CCR5, as a positive control; *B)* control THP-1; *C)* THP-1 plus 16 h GM-CSF. The point at which MIP-1α was added is indicated by an arrow. This figure is representative of 3 separate experiments. The Y axis shows the intensity of Fura-2-AM emission at 500 nm.

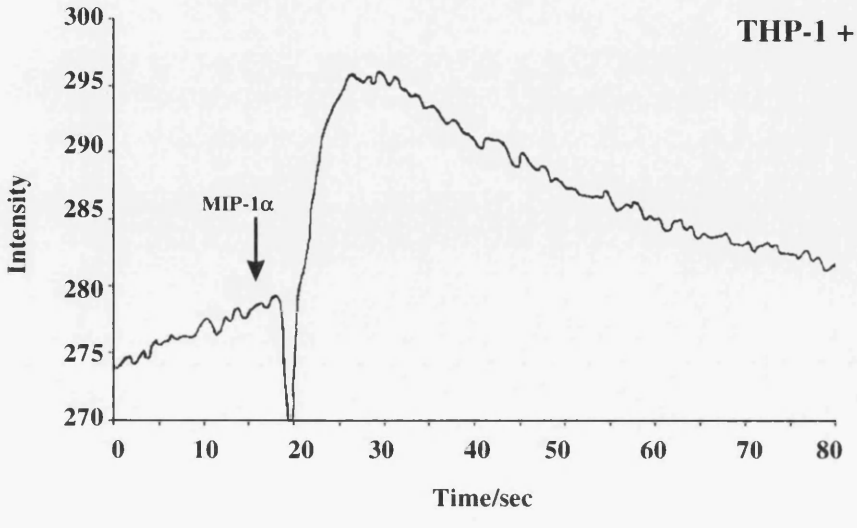
A


HEK-293.hCCR5
Intensity
MIP-1α
450
440
430
420
410
400
0
10
20
30
40
50
60
70
80
Time/sec

B


THP-1 Control
Intensity
MIP-1α
300
295
290
285
280
275
270
0
10
20
30
40
50
60
70
80
Time/sec

C


THP-1 + 16h GM-CSF
Intensity
MIP-1α
300
295
290
285
280
275
270
0
10
20
30
40
50
60
70
80
Time/sec

several unusual phenotypic abnormalities, many of which involve the actions of macrophages. Perhaps the most striking of these, is the macrophage infiltration and defects associated with the abnormal development of the mouse eye in GM-CSF transgenic mice (Cuthbertson and Lang 1989). In GM-CSF transgenic mice, macrophages are autostimulated and produce elevated levels of IL-1α, bFGF and TNF (Lang *et al* 1992). In addition to the ocular defects, GM-CSF transgenic mice also exhibit infiltration of inflammatory cells into the pleural and peritoneal cavities and within striated muscle. As discussed in the introduction, MIP-1α is clearly an important mediator of both haemopoietic and inflammatory functions and is known to be expressed in many disease states, particularly within the lungs. Fahey *et al* (1992) also previously demonstrated that MIP-1α was capable of increasing the expressing of proinflammatory cytokines, such as IL-1 and TNF. The finding that GM-CSF is a potent stimulator of MIP-1α expression in macrophages, together with the importance of macrophages as a source of MIP-1α and the predominant involvement of macrophages in the various pathologies observed in GM-CSF transgenic mice, suggests that MIP-1α might be expressed at an elevated level in these GM-CSF transgenic mice and maybe involved in the migration of monocytes and macrophages in this animal model. It is therefore possible that MIP-1α could be involved in the pathologies observed in these transgenic mice. The expression of MIP-1α in selected tissues of wild type and GM-CSF transgenic mice was therefore examined, to determine if the expression of MIP-1α was elevated in transgenic mice relative to wild type mice.

6.11.2 RT-PCR analysis of MIP-1α expression in GM-CSF transgenic tissues

Using mRNA isolated from tissue samples frozen in liquid nitrogen immediately following excision, the highly sensitive technique of RT-PCR analysis was used to investigate the expression of MIP-1α mRNA in eye, bone marrow, leg muscle and peritoneal cells derived from wild type and GM-CSF transgenic mice. The expression of the house keeping gene β-Actin was also examined in parallel, in an attempt to determine the relative amount of RNA input in each reaction. To allow any observed changes in expression to be more accurately assessed and observed during the log phase of amplification, samples were analysed after 15, 20, 25, 30 and 35

cycles by agarose gel electrophoresis and subsequent Southern blotting of gels. Densitometric analysis was performed on the resulting autoradiographs and the level of MIP-1α expression determined in GM-CSF transgenic samples, relative to wild type samples and to the level of β-Actin expression observed.

6.11.3 MIP-1α expression appears elevated in the eyes of GM-CSF transgenic mice

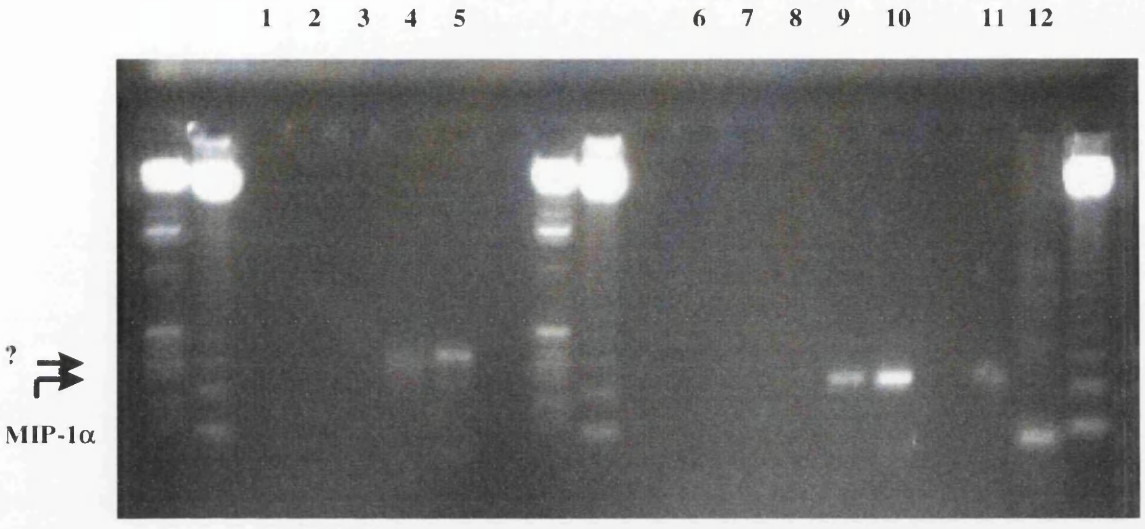
Panel A of figure 6.26 shows the expression of MIP-1α in wild type and transgenic mouse eye, as detected by RT-PCR analysis, whilst panel B below shows the expression of β-Actin. RT-PCR analysis revealed that expression of MIP-1α was only barely visible in wild type eye after 35 cycles of PCR, although an additional band approximately 120 bp larger than expected appeared to be amplified in the wild type eye after 30-35 cycles (indicated by the arrows in figure 6.26, panel A; lanes 4 and 5). Expression of bands of the expected size, but not of the larger size observed in the wild type eye, were however clearly visible in the GM-CSF transgenic eye after 30 to 35 cycles (see figure 6.26, panel A; lanes 9 and 10; position indicated by lower arrow). Also included was a BMM RNA positive control for MIP-1α (figure 6.26, panel A; lane 11). Figure 6.26, panel B; shows the expression of β-Actin to be very similar between the wild type and transgenic samples. The weak, but nevertheless detectable, expression of MIP-1α observed in the wild type eye, was highly unexpected and the source of this expression remains unknown.

Figure 6.27 shows a subsequent Southern blot analysis of the gels shown in figure 6.26, with probes specific for MIP-1α (panel A) and β-Actin (panel B). Analysis of these blots, in conjunction with the results shown in figure 6.26, revealed several things. Firstly, the MIP-1α specific probe appeared only to hybridise to the band corresponding to the expected size of MIP-1α and the larger band observed in the wild type eye by RT-PCR analysis was not detectable, even after prolonged exposure of the autoradiographs (see figure 6.27, panel A; lanes 4 and 5). Secondly, in support of the similar observation made in the RT-PCR analysis, the level of MIP-1α expression appeared to be elevated in the GM-CSF transgenic eye when compared to that of the wild type (see figure 6.27, panel A; compare lanes 9 and 10 with those of lanes 4 and 5). Thirdly, some hybridisation was visible to bands corresponding to the

Figure 6.26: RT-PCR analysis of MIP-1α expression in wild type and GM-CSF transgenic mouse eye.

Panel A, shows the results of an RT-PCR analysis for the detection of MIP-1α mRNA, resolved by agarose gel electrophoresis. The arrow to the left of the gel indicates the position of the MIP-1α mRNA product (expected size of ~267 bp), whilst the arrow above this, marked with a **?**, indicates the relative position of a second unidentified band. Lanes 1-5 show analysis of wild type mouse eye and 6-10 that of GM-CSF transgenic eye. They represent PCR reactions performed for 15, 20, 25, 30 and 35 cycles respectively. Lane 11 is a positive control for MIP-1α, that shows a reaction performed with BMM RNA, whilst lane 12 shows a positive control for the PCR reaction performed using genomic DNA. *Panel B*, shows an identical analysis, performed in parallel, using primers specific for the detection of β-Actin. The lane assignments for lanes 1-10 are as indicated for *A* above. This figure represents a single experiment.

A



B

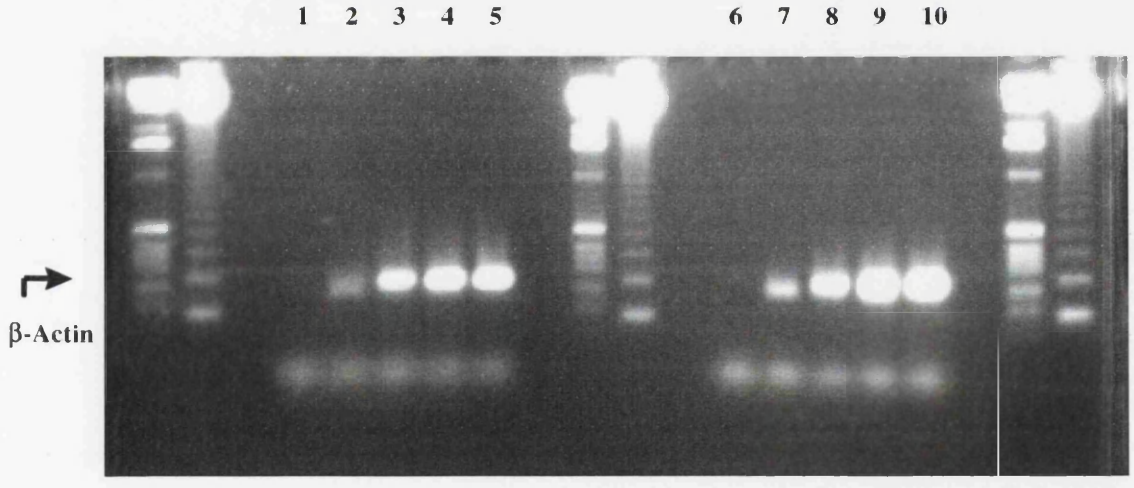
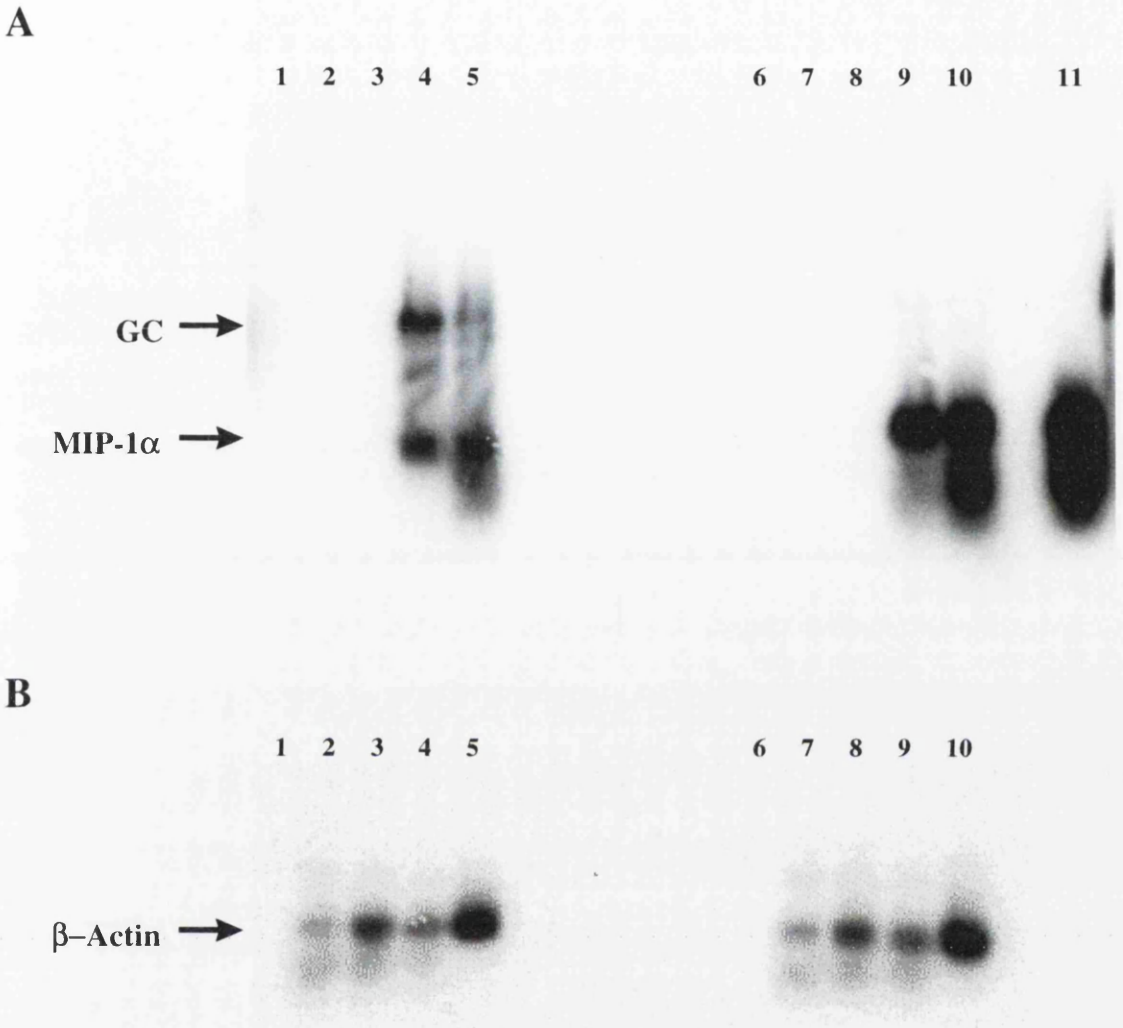


Figure 6.27: Southern blot analysis of MIP-1α and β-Actin expression detected by RT-PCR analysis, in wild type and GM-CSF transgenic mouse eye.

Southern blot analyses were performed on the agarose gels shown in figure 6.26. *Panel A*, shows a blot hybridised with a probe for MIP-1α. The lane assignments for lanes 1-11 are exactly as in the legend for figure 6.26. The lower arrow to the left of the blot indicates the position of the MIP-1α band, the upper arrow (marked with GC) indicates the expected size of genomic DNA (~1 Kb). *Panel B*, shows a blot hybridised with a probe for β-Actin. The lane assignments for lanes 1-10 are as indicated in the legend for figure 6.26. The numbers in the table below represent an approximation of the relative levels of MIP-1α product, as assessed by scanning densitometric analysis and normalisation to calculated β-Actin levels. They represent data from a single experiment.

	Wild Type	**GM-CSF transgenic**
30 Cycles	1.0	2.14
35 Cycles	1.0	5.50


A
1 2 3 4 5
6 7 8 9 10 11
GC
MIP-1α
B
1 2 3 4 5
6 7 8 9 10
β–Actin

expected size of genomic DNA, suggestive of contamination (see figure 6.27 panel A lanes 4 and 5). Indeed, very weak expression of bands corresponding to the expected size of genomic MIP-1α were visible on the original gel, though these are not in fact visible in the image shown in figure 6.26. Figure 6.27; panel B, shows that expression of β-Actin was similar between wild type and GM-CSF samples, suggesting that the initial amount of RNA input to the RT-PCR reactions was very similar. However, it should be noted that the observed expression of β-Actin, as shown in figure 6.27, does not exactly reflect that observed in the gel analysis shown in figure 6.26. It is possible that this reduction may be the result of incomplete transfer of DNA from the gel, during the blotting process. Densitometric analysis showed that this increase in MIP-1α expression was between approximately 2 and 6-fold. Attempts to sub-clone and sequence the larger sized band observed only in the wild type eye, proved unsuccessful and therefore the exact nature of this unidentified band remains unknown.

In summary, these results suggested that the expression of MIP-1α mRNA may be elevated in the eyes of GM-CSF transgenic mice.

6.11.4 MIP-1α expression may be elevated in the bone marrow of GM-CSF transgenic mice

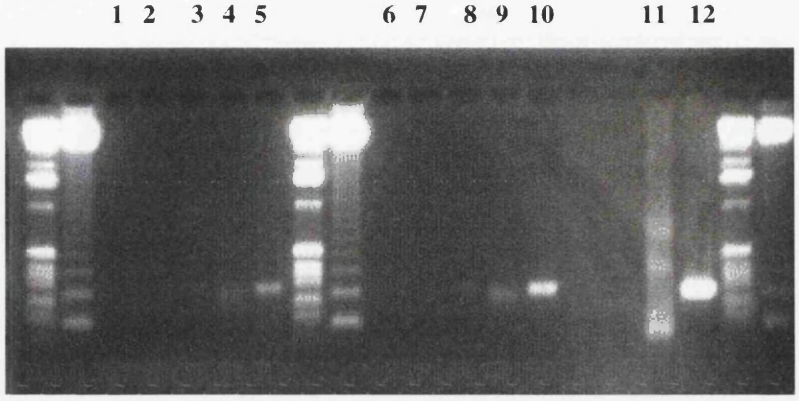
Insufficient bone marrow material was available to allow, either the derivation of BMM, or the sorting of cell populations from total bone marrow. Therefore, the expression of MIP-1α was examined by RT-PCR analysis using total bone marrow cells derived from wild type and GM-CSF transgenic mice, in a manner identical to the analysis performed in section 6.11.3. Products were resolved as before, by agarose gel electrophoresis and figure 6.28 shows the results of this analysis. Panel A shows the expression of MIP-1α, whilst panel B shows that of β-Actin. Expression of MIP-1α was just visible in wild type bone marrow after 30 cycles of PCR and was clearly visible after a further 5 cycles (figure 6.28, panel A; lanes 4 and 5). In GM-CSF transgenic bone marrow this expression appeared to be very slightly elevated relative to the expression observed in wild type samples, with a small increase after 30 cycles and a more visibly evident increase after 35 cycles (see figure 6.28, panel A; compare lane 4 with lane 9, and lane 5 with lane 10). Also included was a genomic control

(lane 11) and a positive PCR control (lane 12). It should also be noted that some genomic MIP-1α expression appears evident in wild type bone marrow samples, though this is not evident from the gel as reproduced here. The presence of this may have resulted in sequestration of reaction components and thus a reduced amplification relative to that of GM-CSF transgenic samples. Panel B of figure 6.28 shows that the expression of β-Actin was not the same in wild type and GM-CSF transgenic samples and the reason for this discrepancy is unclear. Figure 6.29 shows a Southern blot analysis of the same gels as shown in figure 6.28. Unexpectedly, this shows that the greatest level of MIP-1α expression appeared to occur after 30 cycles, and was actually decreased after 35 cycles (see figure 6.29, panel B; lanes 4-5 and 9-10). The expression appeared to be increased in GM-CSF transgenic bone marrow, relative to that observed in the wild type (compare lanes 4 and 9). Lane 11 shows the positive control (previously lane 12 in figure 6.28). Panel B of figure 6.29 shows the expression of β-Actin and clear differences in the pattern of β-Actin expression are evident, compared to that previously observed in panel B of figure 6.28. The reasons for the inconsistencies observed in this experiment are inexplicable and it is therefore difficult to draw any firm conclusions from this data.. It should be born in mind however, that this experiment could only be performed once due to the limited amount of sample material available and these results are therefore clearly not conclusive. It is possible however, that the results obtained from the RT-PCR analysis are indeed correct. If this were the case, then this would indicate that an elevated level of MIP-1α expression may occur in the bone marrow of GM-CSF transgenic mice, compared to that observed for wild type. If true this result would be surprising, in the light of the reported observation that the GM-CSF transgene was not detectable in the bone marrow of GM-CSF transgenic mice (Lang *et al* 1987). However, it should be noted that elevated levels of GM-CSF were previously reported to be detectable in the serum of GM-CSF transgenic mice compared to control mice (Lang *et al* 1987) and this could provide an explanation for any increase in MIP-1α expression that might theoretically occur. Due to the very limited availability of sample material and the inconclusive results obtained, this experiment should clearly be repeated with fresh material.

Figure 6.28: RT-PCR analysis of MIP-1α expression in wild type and GM-CSF transgenic mouse bone marrow.

Panel A, shows the results of an RT-PCR analysis for the detection of MIP-1α mRNA, resolved by agarose gel electrophoresis. The arrow to the left of the gel indicates the position of the MIP-1α mRNA product. Lanes 1-5 show analysis of wild type mouse bone marrow and 6-10 that of GM-CSF transgenic bone marrow. They represent PCR reactions performed for 15, 20, 25, 30 and 35 cycles respectively. Lane 11 shows a positive control for the PCR reaction performed using genomic DNA, whilst the reaction shown in lane 12 is a positive control for MIP-1α performed using MIP-1α cDNA. *Panel B*, shows an identical analysis, performed in parallel, using primers specific for the detection of β-Actin. The lane assignments for lanes 1-10 are as indicated for *A* above. This figure represents a single experiment.

A



B

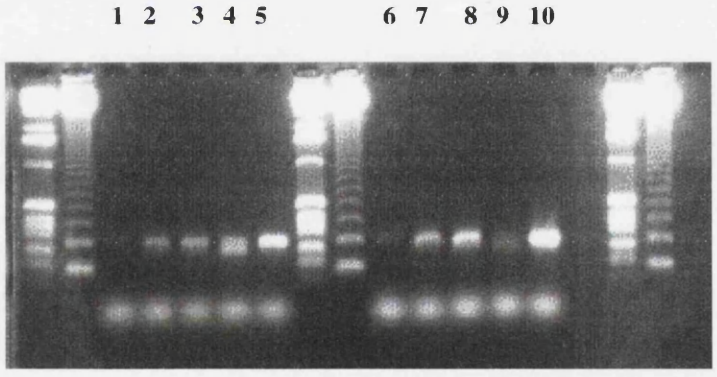
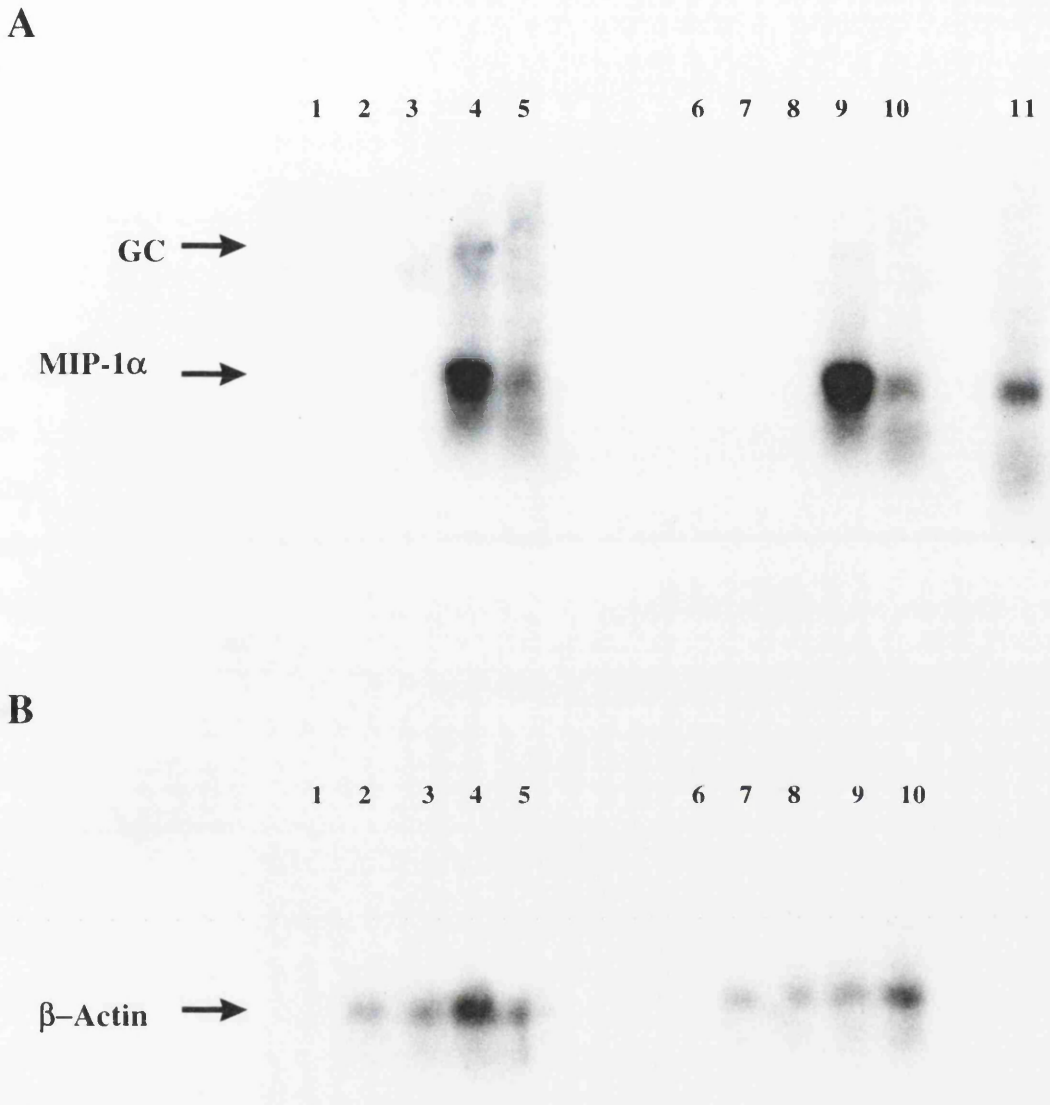


Figure 6.29: Southern blot analysis of MIP-1α and β-Actin expression detected by RT-PCR analysis, in wild type and GM-CSF transgenic bone marrow.

Southern blot analyses were performed on the agarose gels shown in figure 6.28 *Panel A*, shows a blot hybridised with a probe for MIP-1α. The lane assignments for lanes 1-10 are exactly as in the legend for figure 6.28, whilst lane 11 represents the positive control performed using MIP-1α cDNA. The lower arrow to the left of the blot indicates the position of the MIP-1α band, the upper arrow (marked with GC) indicates the expected size of genomic DNA. *Panel B*, shows a blot hybridised with a probe for β-Actin. The lane assignments for lanes 1-10 are as indicated in the legend for figure 6.28. The numbers in the table below represent an approximation of the relative levels of MIP-1α product, as assessed by scanning densitometric analysis and normalisation to calculated β-Actin levels. They represent data from a single experiment.

	Wild Type	GM-CSF transgenic
30 Cycles	1.0	5.60
35 Cycles	1.0	0.60


A
1 2 3 4 5 6 7 8 9 10 11
GC
MIP-1α
B
1 2 3 4 5 6 7 8 9 10
β–Actin

6.11.5 Expression of MIP-1α does not appear elevated in striated muscle or peritoneal cells of GM-CSF transgenic mice

Results obtained from eye samples derived from GM-CSF transgenic mice indicated that MIP-1α expression might be elevated in these mice, compared to wild type mice. Whilst these results were only preliminary and not conclusive, they did however suggest that examination of other tissues was warranted. The expression of MIP-1α was therefore examined in samples of leg muscle and peritoneal cells, since macrophage infiltrates and dramatically increased numbers of cells have been observed in the striated muscle and peritoneal cavities of transgenic mice respectively.

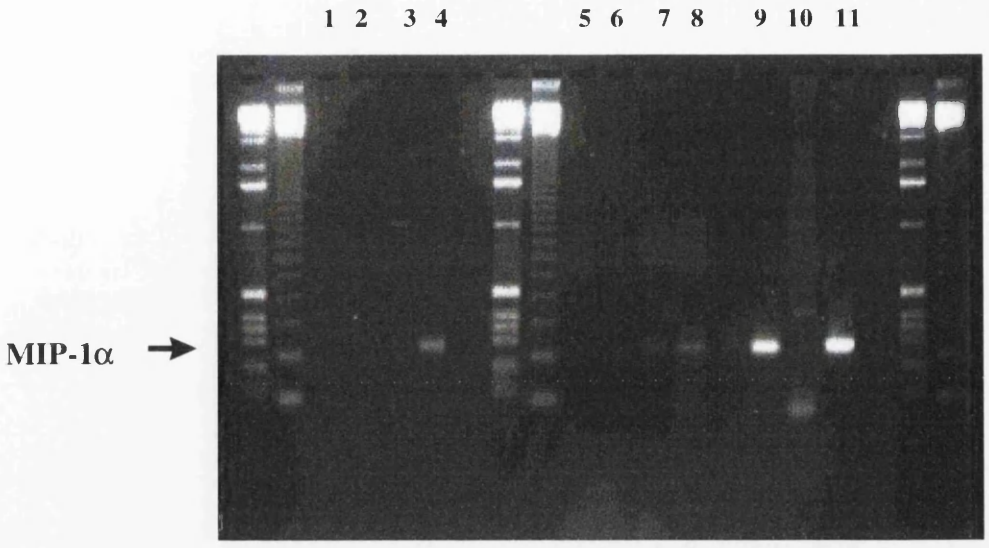
Figure 6.30 shows an RT-PCR analysis of the expression of MIP-1α (panel A) and β-Actin (panel B) in normal and GM-CSF transgenic leg muscle. A similar level of MIP-1α expression was observed in GM-CSF transgenic leg muscle, compared to that of wild type (see figure 6.30, panel A; compare lanes 1-4 with lanes 5-8). Parallel analysis of β-Actin expression revealed that this expression was relatively similar between normal and transgenic leg muscle (see figure 6.30, panel B; compare lanes 1-4 with lanes 5-8). Positive controls were included in lanes 9-11 and 9-10 of panels A and B respectively. A Southern blot of the above experiment confirmed those observations, but also suggested that there was slight contamination of samples with genomic DNA (see figure 6.31) and this was particularly evident in the GM-CSF transgenic leg muscle. The impact of this contamination on a particular RT-PCR reaction can not be assessed, but it remains a possibility that heavy genomic contamination may have sequestered primers and therefore reduced the amplification potential of that particular RT-PCR reaction for available MIP-1α mRNA templates.

Figure 6.32 shows an RT-PCR analysis of the expression of MIP-1α (panel A) and β-Actin (panel B) in normal and GM-CSF transgenic peritoneal cells (PC). Visible MIP-1α expression was only observed in wild type PC after 35 cycles of amplification (see figure 6.32, panel A; lane 5). Parallel analysis of β-Actin expression revealed that the expression was lower in transgenic PC compared to wild type PC (see figure 6.32, panel B; compare lanes 6-10 with lanes 1-5). These differences in β-Actin expression may explain why no MIP-1α expression was observed in the RT-PCR analysis of GM-CSF transgenic PC. Positive controls were included in lanes 11 and 12. Figure 6.33 shows a Southern blot analysis of the same experiment, with panel

Figure 6.30: RT-PCR analysis of MIP-1α expression in wild type and GM-CSF transgenic mouse leg muscle.

Panel A, shows the results of an RT-PCR analysis for the detection of MIP-1α mRNA, resolved by agarose gel electrophoresis. The arrow to the left of the gel indicates the position of the MIP-1α mRNA product. Lanes 1-4 show analysis of wild type mouse leg muscle and 5-8 that of GM-CSF transgenic leg muscle. They represent PCR reactions performed for 20, 25, 30 and 35 cycles respectively. Lane 9 is a positive control for the RT reaction performed using BMM RNA, lane 10 is a positive control for the PCR reaction performed using genomic DNA, whilst lane 11 is another positive control performed using genomic DNA. *Panel B*, shows an identical analysis, performed in parallel, using primers specific for the detection of β-Actin. The lane assignments are as indicated for *A* above, with the exception of positive controls for the PCR performed with genomic DNA (lane 9) and β-Actin cDNA (lane 10). This figure represents a single experiment.

A



B

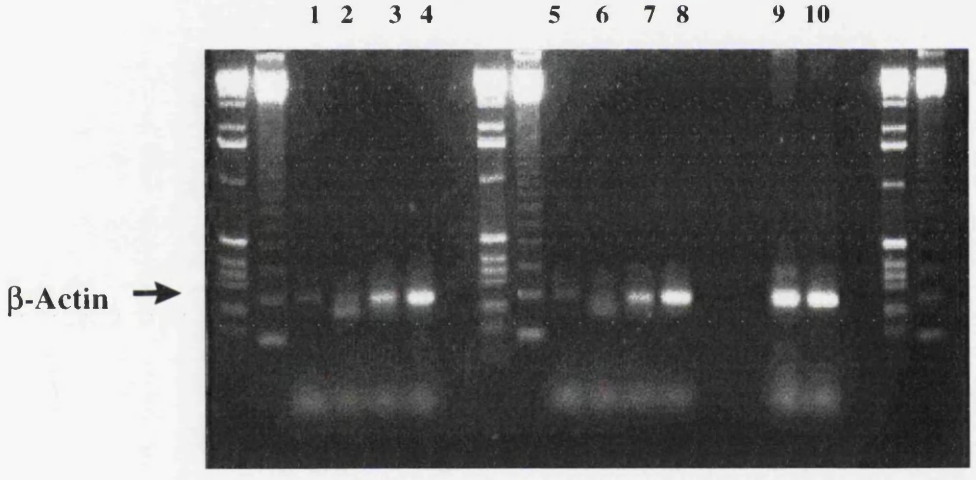


Figure 6.31: Southern blot analysis of MIP-1α and β-Actin expression detected by RT-PCR analysis, in wild type and GM-CSF transgenic leg muscle.

Southern blot analyses were performed on the agarose gels shown in figure 6.12.4.1. *Panel A*, shows a blot hybridised with a probe for MIP-1α. The lane assignments for lanes 1-9 are exactly as in the legend for figure 6.30, with the exception of lanes 10 and 11 which have not been included. The lower arrow to the left of the blot indicates the position of the MIP-1α band, the upper arrow (marked with GC) indicates the expected size of genomic DNA. *Panel B*, shows a blot hybridised with a probe for β-Actin. The lane assignments for lanes 1-8, are as indicated in the legend for figure 6.30. The numbers in the table below represent an approximation of the relative levels of MIP-1α product, as assessed by scanning densitometric analysis and normalisation to calculated β-Actin levels. They represent data from a single experiment.

	Wild Type	GM-CSF transgenic
35 Cycles	1.0	0.60

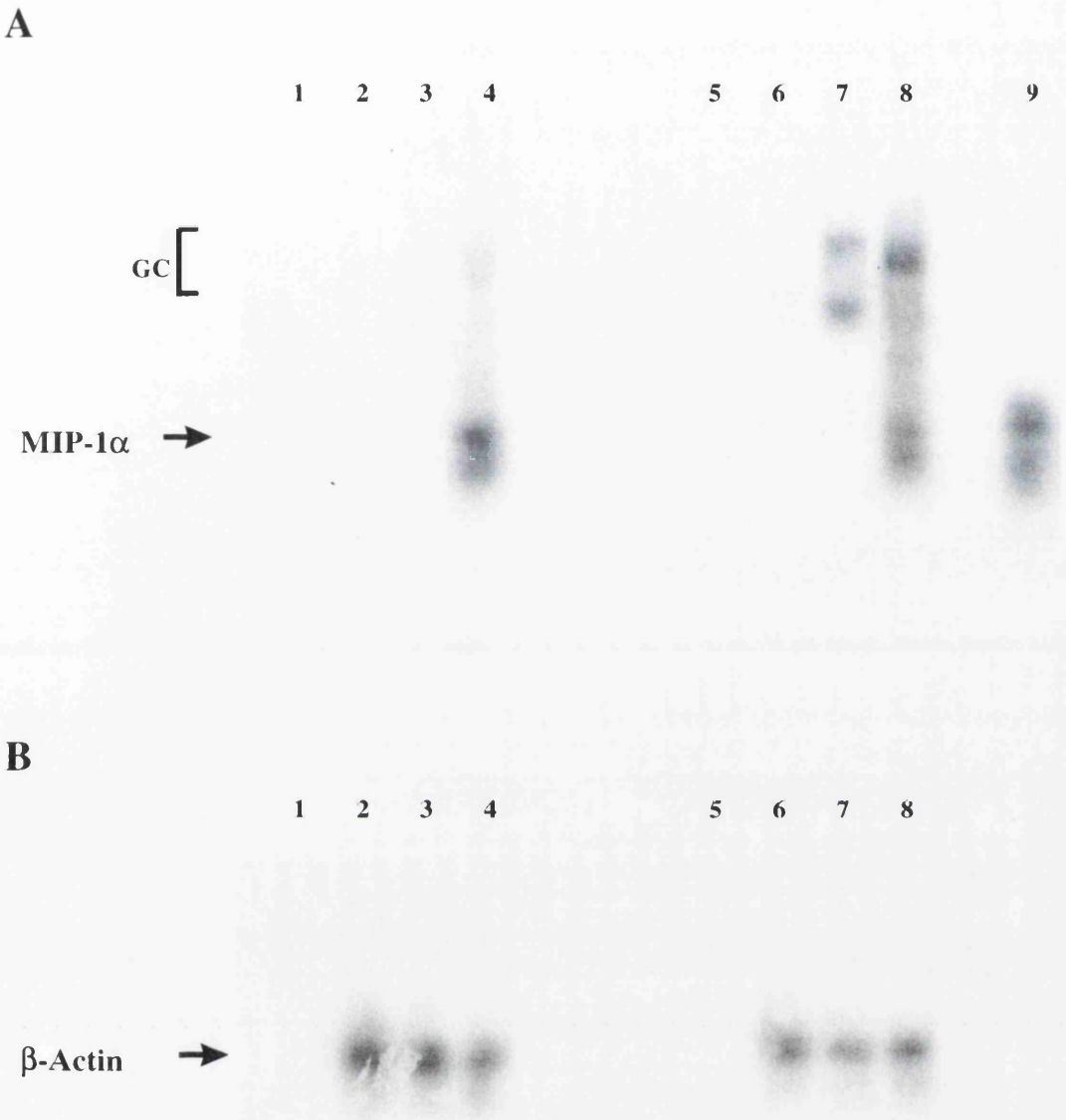
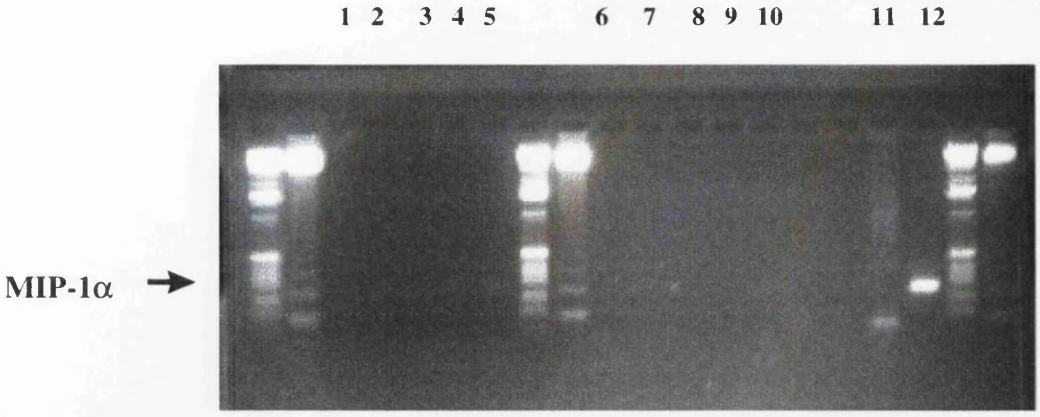

A
1 2 3 4 5 6 7 8 9
GC
MIP-1α
B
1 2 3 4 5 6 7 8
β-Actin

Figure 6.32: RT-PCR analysis of MIP-1α expression in wild type and GM-CSF transgenic mouse peritoneal cells.

Panel A, shows the results of an RT-PCR analysis using primers specific for the detection of MIP-1α mRNA, resolved by agarose gel electrophoresis. The arrow to the left of the gel indicates the position of the MIP-1α mRNA product. Lanes 1-5 show analysis of wild type mouse peritoneal cells and 6-10 that of GM-CSF transgenic peritoneal cells. They represent PCR reactions performed for 15, 20, 25, 30 and 35 cycles respectively. Lanes 11 is a positive control for the PCR reaction performed with genomic DNA, whilst lane 12 shows a positive control reaction for MIP-1α performed using MIP-1α cDNA. *Panel B*, shows an identical analysis, performed in parallel, using primers specific for the detection of β-Actin. The lane assignments for lanes 1-10 are as indicated for *A* above. This figure represents a single experiment.

A



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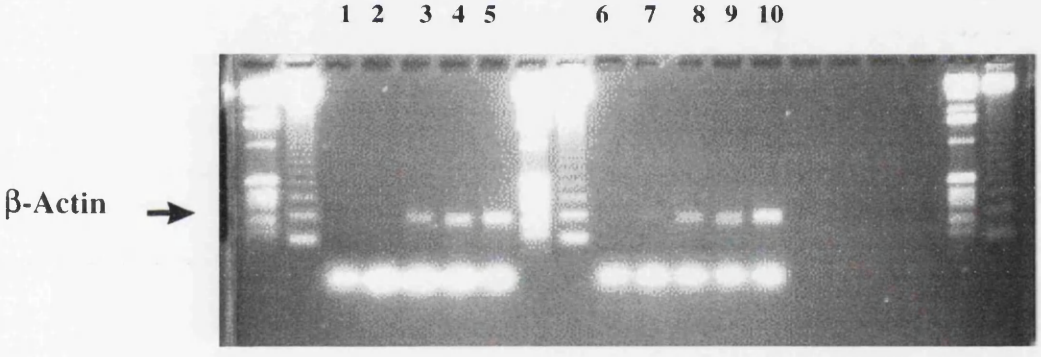
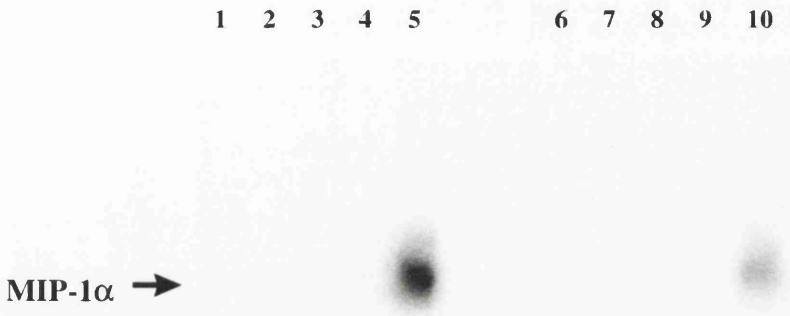


Figure 6.33: Southern blot analysis of MIP-1α and β-Actin expression detected by RT-PCR analysis, in wild type and GM-CSF peritoneal cells.

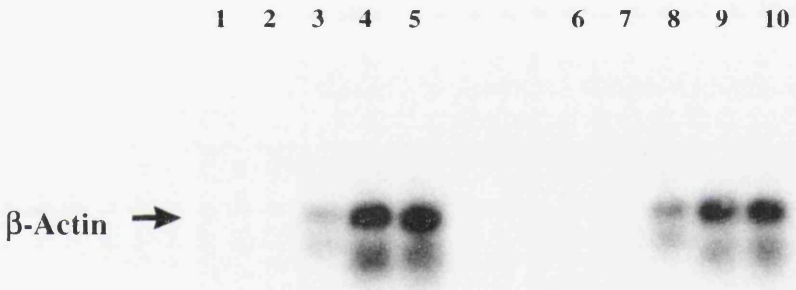
Southern blot analyses were performed on the agarose gels shown in figure 6.32 *Panel A*, shows a blot hybridised with a probe for MIP-1α. The lane assignments for lanes 1-10 are exactly as in the legend for figure 6.32. The arrow to the left of the blot indicates the position of the MIP-1α band. *Panel B*, shows a blot hybridised with a probe for β-Actin. The lane assignments for lanes 1-10 are as indicated in the legend for figure 6.32. The numbers in the table below represent an approximation of the relative levels of MIP-1α product, as assessed by scanning densitometric analysis and normalisation to calculated β-Actin levels. They represent data from a single experiment.

	Wild Type	GM-CSF transgenic
35 Cycles	1.0	0.60

A



B



A showing MIP-α expression and panel B that of β-Actin. This indicates that some expression of MIP-1α was visible in both normal and GM-CSF transgenic PC after 35 cycles, although this GM-CSF transgenic expression was not visible in the RT-PCR analysis as previously shown in figure 6.32. The southern blot analysis of β-Actin expression (as shown in figure 6.33) support the RT-PCR analysis of β-Actin expression, confirming lower β-Actin expression in the GM-CSF transgenic PC compared to wild type PC. This suggests that differences in the original amount of input mRNA may have occurred during the setting-up of the RT-PCR reactions. No genomic DNA contamination was detected in any of the lanes. Densitometic analysis of the levels of MIP-1α expression (see figure 6.33, panel A; compare lane 5 with lane 10) with those of β-Actin expression (see panel B) revealed that essentially the same levels of MIP-1α expression occurred in PC from GM-CSF transgenic mice, as did in PC from normal mice.

In summary, these results indicate that whilst the level of MIP-α expression may be elevated in the eye and bone marrow of GM-CSF transgenic mice, compared to wild type mice, the level does not appear to be elevated in the leg muscle and peritoneal cells. It should be noted, that due to the very limited amount of sample material, these experiments were only performed once and therefore the analysis of the results can not be totally conclusive. They do however suggest that elevated levels of MIP-1α may exist in specific tissues within GM-CSF transgenic mice, compared to normal mice and a more extensive analysis of these observations would perhaps be informative. In the light of the preliminary observations indicated previously in section 6.11, it was decided to further analyse the expression of MIP-1α in GM-CSF transgenic tissues, using the technique of *in situ* hybridisation. Analysis of frozen tissue sections from eye, leg muscle and peritoneal cells was performed using this technique, but unfortunately proved uninformative.

CHAPTER 7: DISCUSSION

7.1 Introduction

One of the most obvious conclusions resulting from the research that has accumulated over the past 20 years or so, into the functions of the multitude of haemopoietic growth factors and cytokines that act upon the immunohaemopoietic system, is the enormous complexity of the regulatory mechanisms in which they are involved. As discussed previously in section 1.8, there exists a large interacting network of cytokines that exhibit a large degree of functional overlap and potential for redundancy. Our laboratory is particularly interested in the role of MIP-1α in the regulation of stem cell proliferation and previous research has attempted to dissect the interactive relationship between MIP-1α and another inhibitor of haemopoietic stem cell proliferation, TGF-β1. As a result of this investigation it is now believed that MIP-1α and TGF-β1 have an endogenous, reciprocal relationship in the bone marrow, in which TGF-β1 probably functions to suppress the expression of MIP-1α (Maltman *et al* 1993, 1996). A study by Cluitmans *et al* (1995) has however, demonstrated the simultaneous presence of both TGF-β1 and MIP-1α in the bone marrow and peripheral blood of normal individuals. Clearly therefore, in addition to suppressive factors such as TGF-β1, the expression of MIP-1α also must be regulated by positively acting factors. To date, our understanding of the regulatory mechanisms controlling the expression of MIP-1α is minimal, particularly with regard to its regulation within the bone marrow. The focus of this thesis has therefore been to attempt to further understand the regulation of MIP-1α expression, with a particular emphasis on its role within this complex network of regulatory cytokines. One very important point that must be considered in such an investigation, is that MIP-1α (as well as many other cytokines involved in this network) functions as both a haemopoietic and inflammatory regulator. Any conclusions resulting must therefore consider both these aspects, as conclusions regarding inflammatory functions may be informative about haemopoietic functions and vice versa. For the purposes of this discussion, although inflammation is a natural part of haemopoiesis, they will be considered as being distinct. Thus in this discussion, the term haemopoiesis will refer to constitutive and inducible haemopoiesis, such as that occurring during the generation of mature end cells. The term

inflammation will be used in the context of 'inflammatory functions', such as histamine release, phagocytosis, etc. and particularly with regard to mature immune cells.

The primary source of MIP-1α within the bone marrow is considered to be the macrophage. Previous studies in our laboratory have successfully used the well characterised bone marrow-derived macrophage (BMM) and this was therefore selected as a cell system in which to examine the regulation of MIP-1α expression. The initial investigations sought to identify haemopoietic cytokines as potential regulators of MIP-1α expression. The results shown in figure 6.1, demonstrate that neither G-CSF, IL-6, nor LIF had any visible effects on the expression of MIP-1α in BMM. Receptors for LIF, G-CSF and IL-6 have all been reported to be expressed on macrophages (Hilton *et al* 1988, Shieh *et al* 1991, Lotem and Sachs 1987. This suggests that the lack of response of BMM to LIF, G-CSF and IL-6 is not due to the absence of expression of receptors for these cytokines. In contrast however, figure 6.2 shows that GM-CSF did affect MIP-1α expression, resulting in a marked increase in the levels of MIP-1α mRNA. Given the many known haemopoietic and inflammatory functions of GM-CSF, particularly with regard to cells of the monocyte / macrophage lineage, this observation was of considerable interest. It was therefore decided to examine the role of GM-CSF and related cytokines in the regulation of MIP-1α expression, in greater detail.

7.2 Induction of MIP-1α Expression by GM-CSF and IL-3

Initial experiments suggested that GM-CSF is a strong inducer of MIP-1α expression and to confirm this observation, further investigation was therefore performed. The results shown in figures 6.3 and 6.4, demonstrate that GM-CSF rapidly induces MIP-1α expression and that this is dose dependent. It is likely that this induction occurs directly, rather than via induction of a secondary cytokine, since the induction is visible within 30 minutes. This is however, merely suggestive and remains to be determined. The observed induction even at a low concentration of GM-CSF, suggests that GM-CSF is relatively potent in its effects. Figure 6.5 demonstrates that the continued presence of either serum or L929 CM is not a prerequisite for the inductive effects of GM-CSF stimulation. This implies that GM-CSF is not synergising with additional factors present in the serum or L929 CM. Kasama *et al*

(1993) previously demonstrated that GM-CSF was able to synergise with LPS to increase the level of MIP-1α expression in neutrophils, but that GM-CSF alone was unable to induce MIP-1α expression over a wide range of concentrations. This is in contrast to the observations shown in figures 6.2 to 6.5, in which GM-CSF rapidly induces MIP-1α expression over a wide range of concentrations. The fact that addition of a GM-CSF-specific neutralising antibody resulted in the substantial abolishment of the GM-CSF induced expression of MIP-1α expression, shows that GM-CSF was indispensable for this induction. Furthermore, because these experiments were carried out under conditions which were designed (as far as possible) to be essentially LPS free, it is therefore unlikely that LPS plays a role in this observed induction of MIP-1α mRNA by GM-CSF. The contrast in these results to those of Kasama *et al* (1993), suggest that clear differences exist between neutrophils and macrophages in the GM-CSF regulated expression of MIP-1α expression. It should also be noted that their study was performed using human neutrophils, whilst the macrophages used in this study were murine in origin. Although the promoters of murine MIP-1α and the various forms of human MIP-1α (LD78) are similar (Widmer *et al* 1991), with approximately 75 % homology within the first 350 bp of proximal promoter sequences, it is possible that differences may exist in the regulatory control of MIP-α and LD78 acting in the two species. If so, this could be one explanation for the observed differences between this study and that of Kasama *et al* (1993).

It is recognised that increases in mRNA expression are not always followed by a corresponding increase in protein expression, which casts doubt on the physiological and functional relevance of any changes in mRNA levels that are not reflected at protein level. Efforts were therefore made to examine the expression of MIP-1α protein, following GM-CSF stimulation. The observation that MIP-1α protein was only detectable following concentration of BMM supernatants (see figure 6.7) was in keeping with previous data from our laboratory by Maltman *et al* (1993) who previously reported that the expression of MIP-1α protein in unconcentrated BMM medium was undetectable using Western blot analysis. Whilst it is unclear whether or not full translation of MIP-1α mRNA into protein occurs in BMM following GM-CSF stimulation, the level of protein seems low compared to the level of mRNA. It is theoretically possible therefore that for full translation of mRNAs to occur, an

additional signal is required. Indeed there is evidence that such mechanisms may operate for the CC chemokine RANTES and other cytokines, during the induction phase of T cell anergy (Schall *et al* 1992). Certainly one can speculate that it may be desirable to limit expression of a protein with potent inflammatory effects, such that this expression only occurs when strictly needed. Indeed one can stretch this hypothesis to suggest that expression could be limited to specific niches and locations. One possible hypothetical mechanism for such control, could be through the action of adhesion molecules and other matrix associated molecules. Expression of these could be limited to particular niches or circumstances, such that only when a cell is interacting with them, do they provide the necessary secondary stimulus. Therefore, in the context of GM-CSF stimulation (for example in the immediate locale of a inflammatory infection), GM-CSF would act to prime cells for increased expression of MIP-1α, but this would only occur following the appropriate secondary signal. It is clear however, that some increased protein expression does result from GM-CSF stimulation and suggests that regulation of MIP-1α expression by GM-CSF is physiologically relevant.

As indicated in the introduction, GM-CSF is in fact a member of a small subfamily of cytokines and shares extensive functional homology with IL-3, as well as the shared usage of the common β-subunit in their respective receptors. In fact GM-CSF and IL-3 receptors have previously been suggested to be co-ordinately regulated in macrophages (Fan *et al* 1992), providing further evidence for linkage in their actions. The reported overlap in specific functions of GM-CSF and IL-3 suggested that IL-3 might also therefore be a candidate regulator of MIP-1α expression. Figures 6.8 A and 6.8 B, demonstrate that IL-3 did indeed appear to exert a very similar effect upon MIP-1α expression, as did GM-CSF. The kinetics of this expression were identical and as with GM-CSF, the dose of IL-3 required to exert a visible increase in MIP-1α expression was low. Thus, both GM-CSF and IL-3 appear to be strong inducers of MIP-1α expression. One interesting possibility, reported during the writing of this thesis, was evidence for the expression of a pre-formed human GM-CSF receptor complex that could be activated by GM-CSF, IL-3 or IL-5 (Woodcock *et al* 1997). This suggests that it is theoretically possible that the observed induction of MIP-1α expression by IL-3 could be occurring through such a pre-formed receptor

complex, rather than through the expected IL-3 receptor complex., GM-CSF As indicated below, IL-5 was unable to effect MIP-1α expression, which suggests that this pre-formed receptor complex is not present on BMM. However it is unknown if a similar preformed murine receptor complex actually exists.

7.3 Effect of IL-5 on MIP-1α Expression

Given the shared ability of GM-CSF and IL-3 to induce MIP-1α expression, their shared usage of the common β subunit and because IL-5 is a member of this same subfamily, with some similar functions and that it also signals through the common β subunit, it was considered a possibility that IL-5 may also be able to regulate MIP-1α expression, in a similar fashion to that observed for GM-CSF and IL-3. However, the results presented in figures 6.9 to 6.11, suggest that IL-5 does not regulate MIP-1α expression in macrophages, or other cells within the murine bone marrow. That IL-5 does not effect MIP-1α expression in macrophages is perhaps obvious and is clearly due to the absence of functional IL-5 receptors. Indeed, Takagi *et al* (1995) previously reported that the lineage specificity of IL-5 was mainly due to the limited expression of IL-5Rα chain. Results not presented in this thesis have suggested that IL-5 has no apparent effect on MIP-1α expression in murine B cells lines. It is possible however, that in the B cell lines examined MIP-1α may be transcriptionally silent. In hindsight, a more appropriate model would perhaps have been to analyse the expression of MIP-1α in primary human eosinophils. Attempts were in fact made to perform this analysis, though they were unsuccessful, due to the logistical difficulties involved in obtaining samples, particularly from patients with eosinophilia. Therefore, the question of whether IL-5 functions as a regulator of MIP-1α expression remains unanswered. Recent evidence from a report by Li *et al* (1996) does suggest however, that IL-5 may have some limited effects on MIP-1α expression in basophils, though this was reported to be not significant.

7.4 Induction of other CC chemokines by GM-CSF and IL-3

Previously, Takahashi *et al* (1993) showed that in monocytes, both GM-CSF and IL-3 were potent inducers of IL-8 expression, another member of the chemokine superfamily. This suggested the possibility that additional members of the chemokine

superfamily might also be regulated by GM-CSF and IL-3. As can be seen in figures 6.12 and 6.13, expression of the CC chemokines MIP-1β, JE and MARC were all elevated following GM-CSF and IL-3 treatment. Current evidence suggests that that both JE/MCP-1 and MARC/MCP-1 are functionally important as inflammatory cytokines (Huffnagle *et al* 1995, Stafford *et al* 1997). This would suggest that their induction by GM-CSF and IL-3 is related to a role in inflammatory functions, though it should be noted that this does not preclude the involvement of GM-CSF and IL-3 in regulating MIP-1α expression in the context of haemopoiesis. Several studies have attempted to define regulatory elements involved in controlling the expression of various chemokine genes, such as MIP-1α, MCP-1, RANTES and MARC (Grove and Plumb 1993, Ray *et al* 1997, Moriuchi *et al* 1997, Jarmin *et al* 1994). Current evidence suggests that many of these genes may share the presence of common transcription factor binding sequences within their promoters. It is certainly feasible therefore, that factors such as NF-κB, previously identified as being potentially involved in regulating the expression of MIP-1α and other CC chemokines (Grove and Plumb 1993, Moriuchi *et al* 1997, Ueda *et al* 1997), may be involved in the regulation of other genes within the same family. If this were indeed the case, it is certainly possible to hypothesise that one important function of GM-CSF and IL-3, may be to non-specifically upregulate chemokine expression. As discussed earlier, this may relate to a potential priming role of GM-CSF and IL-3. The regulatory control of the GM-CSF gene appears to be fairly complex. For instance in T cells, multiple signals appear to be required for GM-CSF expression to occur and it is evident that elements containing binding motifs for transcription factors such as ETS1, AP1, NFAT, NFATp and NF-κB and NF-κB-like are just a few of those that have been implicated in the regulation of GM-CSF expression in a variety of cell types (Jenkins *et al* 1995, Thomas *et al* 1997). Indeed, mice deficient in c-*rel* (a subunit of the NF-κB-like family of transcription factors) exhibit defects in the production of GM-CSF and IL-3 (Gerondakis *et al* 1996), underlining the importance of this family of transcription factors in the regulation of GM-CSF and IL-3 expression. Also of interest is the observation that some of these factors have been shown to be involved in the regulation of chemokine expression. Examples include NFAT, in the control of MARC expression in allergically triggered murine mast cells (Jarmin *et al* 1994,

Prieschel *et al* 1995) and as already mentioned above, NF-κB in the regulation of other chemokines. If these transcription factors are involved in the regulation of both GM-CSF or IL-3 and the genes they induce, then this could provide additional amplification.

Interestingly, direct support for the observation that GM-CSF and IL-3 were potent inducers of selective chemokine gene expression in BMM had been previously reported, in a very similar study by Orlofsky *et al* (1994). In this report, the authors investigated the effects of GM-CSF on expression of the CC chemokine C10 in BMM and also noted that expression of MIP-1α, JE and RANTES was induced in BMM. There are several discrepancies observed, between results reported in this study and that of Orlofsky *et al* (1994). They reported that expression of MIP-1α was only weakly induced by GM-CSF, whilst in contrast the results reported in this work show that expression of MIP-1α mRNA was markedly induced by GM-CSF. One possible explanation for this is that Orlofsky *et al* stimulated their BMM for 24 h with GM-CSF, whereas the results reported herein were achieved with only 4 h stimulation. The results discussed in section 6.3.2 suggest that MIP-1α expression had declined within 6 h, following GM-CSF stimulation. It is therefore quite possible that expression of MIP-1α in response to GM-CSF had declined by 24 h, as reported by Orlofsky *et al.* Another contrast, is that in this study no expression of RANTES was detectable in BMM, in concordance with the previous study of Maltman *et al* (1996), whilst Orlofsky and colleagues reported that RANTES was also induced by GM-CSF. However, they did acknowledge that this induction could have been due to LPS contamination. During the course of all experiments performed and reported in this thesis, precautions were taken to ensure that as far as was possible, no LPS was present. The authors also reported similar effects by IL-3, but presented no data. Whilst some discrepancies exist between this study and that of Orlofsky *et al*, probably due to experimental differences, they do both suggest that GM-CSF and IL-3 are stimulators of chemokine expression. Moreover, the results presented herein extend previous observations, to include the novel observation that MIP-1β and MARC are both induced by GM-CSF and IL-3. Whilst it is probable that the widespread expression of chemokines in response to GM-CSF and IL-3 is likely to be the result of a role in an inflammatory context, confirmation of this may only occur

when the effects of GM-CSF and IL-3 on the expression of a much wider range of chemokines are examined. In fact one such novel chemokine, known as MPIF-2, was recently cloned and reported to be weakly induced in GM-CSF-treated macrophages (Patel *et al* 1997), providing further evidence that this GM-CSF/IL-3 mediated induction may be relatively widespread amongst members of the chemokine superfamily.

7.5 TGF-β1 Suppression of Members of the MIP-1α Superfamily

As discussed in section 3.6.2, previous work in our laboratory has discovered that an endogenous, reciprocal relationship exists in BMM, between TGF-β1 and MIP-1α. In the light of the findings raised during the course of the work reported in this thesis, the effect of TGF-β1 on GM-CSF and IL-3 induced MIP-1α expression was therefore of significant interest. The results in figures 6.14 and 6.15 confirmed that TGF-β1 was a suppressor of GM-CSF and IL-3 induced MIP-1α expression. This suppression was however not complete, suggesting that GM-CSF and IL-3 might be sufficiently potent in their effects to overcome the endogenous block exerted by TGF-β1 on MIP-1α expression. However, it should be noted that it is also equally possible to speculate the reverse, namely that TGF-β is sufficient enough to at least partially overcome the inductive stimulus of GM-CSF or IL-3. Either of these may hold true, and they are not necessarily exclusive. This finding was however of significant interest, given the previous report of Cluitmans *et al* (1995), suggesting that some MIP-1α expression was detectable in the bone marrow, despite the presence of TGF-β1. It is therefore possible that *in vivo* GM-CSF and IL-3 may act to overcome this TGF-β1 mediated block. However, the relationship between TGF-β, GM-CSF, IL-3 and MIP-1α *in vivo*, is likely to be dynamic and the precise level of MIP-1α expression may therefore depend upon the relative levels of GM-CSF, IL-3 and TGF-β at any given time. Indeed, the constitutive expression of GM-CSF within the haemopoietic microenvironment has been reported to be somewhat variable. Some reports have suggested that constitutive GM-CSF expression does not occur (Cluitmans *et al* 1995), whilst others have documented expression of GM-CSF by adherent cells within bone marrow cultures (Charbord *et al* 1991). Clearly however, GM-CSF is expressed by a wide variety of cell types and in response to a variety of

inducing stimuli. More certain is the observation that constitutive IL-3 expression is undetectable. However, as discussed previously in the introduction, it is possible that such expression may occur in localised niches within the microenvironment or following conditions of extreme stress.

The observations made in figure 6.16, that TGF-β1 also suppressed JE and MARC expression following GM-CSF stimulation suggests, that the suppressive effect of TGF-β1 is more widespread and therefore probably represents the well recognised ability of TGF-β1 to function as an immuno-suppressive factor. However, this observation does not preclude the possibility that one role of TGF-β1 may be to suppress the induction of chemokine expression in the bone marrow microenvironment, under non-inflamed conditions.

7.6 Expression of CCR1 in BMM is Regulated Differently to that of CCR5, in Response to GM-CSF and IL-3

Recently, a greater understanding of the interaction of chemokines with their cognate receptors has been achieved. One of the most important reasons for this recent increase in understanding, has been due to the explosion of interest in the roles of chemokines and chemokine receptors in the progression of HIV pathogenesis. However, one area which has remained poorly understood is that of the regulation of chemokine receptor expression. Understanding this is clearly important, not just for novel aspects of chemokine biology such as HIV infection, but also for understanding inflammatory and haemopoietic aspects of chemokine function.

The data shown in figures 6.17 to 6.19 show that the expression of CCR1 is induced by GM-CSF and IL-3 in BMM and splenic cells, whilst CCR5 expression is unaltered. This suggests that the expression of CCR1 and CCR5 is regulated differently. One inference from these results, is that CCR1 and CCR5 may have distinct functions. It is possible that expression of CCR5 may be under a more restricted and constitutive control. The reason for this different regulation of expression is unclear, but it may reflect some importance of CCR1 over that of CCR5, under conditions in which GM-CSF or IL-3 are expressed (i.e. under inflammatory conditions). Indeed evidence from recent studies in which CCR1 was deleted in mice, have provided clear evidence that CCR1 has a crucial inflammatory role *in vivo* (Gao *et al* 1997, Gerard *et al* 1997). Attempts to demonstrate a functional significance of

this increase in CCR1 expression, by analysis of potential changes in calcium mobilisation, were unsuccessful. However as discussed in the results, this remains to be determined and may reflect experimental difficulties, rather than a lack of effect of GM-CSF on CCR1 expression, in a functional context. The effect of TGF-β1 on CCR1 and CCR5 expression appears to be minimal, apparently only affecting the basal level of expression. The significance of this observation is unclear. Different mechanisms for the regulatory control of chemokine receptor expression is not a new observation and several reports were published during the course of these studies, demonstrating that IL-2 regulated the expression of CCR1, CCR2 and CCR5, but did not alter the expression of CXCR1, CXCR2 or CXCR4 (Loetscher *et al* 1996b, Bleul *et al* 1997). Several reports have also documented suppressive effects of various agents, such as LPS, IFN-γ, TNF-α and IL-1α on chemokine receptor expression (Sica *et al* 1997, Tangirala *et al* 1997). In fact, Tangirala *et al* reported that treatment of THP-1 cells with GM-CSF resulted in a marked reduction in the expression of hCCR2 mRNA. Interestingly, in this same study the authors also reported that differentiation of THP-1 cells with phorbol myristate acetate (PMA) resulted in an almost complete loss of hCCR2 mRNA expression. As indicated in figure 6.19 B, GM-CSF did not appear to affect mCCR2 expression. The reason for the difference between this finding and that reported by Tangirala *et al* is unclear, but may be due to the different cell systems used and perhaps the difference in differentiation state of BMM compared to THP-1. A recent report, published during the course of writing this thesis, has provided further and intriguing evidence of the potential complexity of chemokine receptor expression. This report by Mummidi *et al* (1997), provided evidence for multiple transcripts for human CCR5 (derived by alternative splicing and usage of multiple transcriptional start sites) and the usage of multiple promoters. Previous reports have documented alternative splicing for CCR2 (Charo *et al* 1994), and CXCR4 (Heesen *et al* 1997), suggesting that a complex regulation of gene expression and organisation may occur in other chemokine receptors. Future investigation of the promoters of the various chemokine receptor genes will no doubt yield further understanding of the regulation of their expression. In the context of the results presented in this thesis, a comparative analysis of the promoters for murine CCR1 and CCR5 following GM-CSF treatment would be a very useful approach to further understanding why they appear to be differently regulated.

No stem-cell inhibitory specific receptor has been reported for MIP-1α as yet, and current evidence from our laboratory suggests that the inhibitory actions of MIP-1α are not mediated through any of the reported MIP-1α receptors (Graham *et al* 1996). It is probable that the observed effects of GM-CSF and IL-3 on CCR1 expression as reported in this work, relate to involvement in an inflammatory mechanism. Further understanding and confirmation of this must however, await identification of this stem cell inhibitory receptor.

7.7 Analysis of MIP-1α Expression in GM-CSF Transgenic Mouse Tissues

The studies discussed in the previous sections, strongly suggest a potent effect of GM-CSF and IL-3 on MIP-1α expression *in vitro*. However they have provided little insight into any role of GM-CSF or IL-3 *in vivo*. As discussed in various sections in the introduction, analysis of knock out and transgenic mice have in many cases proved a useful approach to further understanding the potential *in vivo* role(s) of growth factors and cytokines. As discussed previously in section 2.12, analysis of mice overexpressing GM-CSF, as performed by Lang *et al* (1987), revealed some unusual phenotypic abnormalities particularly with regard to macrophage function. It was speculated by us, that the levels of MIP-1α might be elevated in these mice as a result of over expression of GM-CSF and indeed increased expression was previously reported for other GM-CSF inducible growth factors in these transgenic mice (Lang *et al* 1992). MIP-1α may therefore be ultimately responsible for aspects of the GM-CSF transgenic phenotype. It was therefore decided to perform a brief examination of the expression of MIP-1α in these mice.

It should be pointed out here, that only a very limited amount of material was available for examination and therefore any investigation is, by necessity, of a purely preliminary nature. However, as can be seen in figures 6.26 to 6.29, RT-PCR analysis and subsequent Southern blot analysis did suggest that the level of MIP-1α expression was elevated in the eye, and perhaps also in the bone marrow, of a GM-CSF transgenic mouse, relative to that observed in a wild type littermate control. The precise source of this MIP-1α in wild type mice eyes is unknown, but may result from MIP-1α expressing cells within the vasculature of the eye or perhaps even infiltrating leukocytes. The elevated expression in the eye of the transgenic mouse was

unsurprising, given the potent effect of GM-CSF on MIP-1α expression. It must be pointed out that this observed increase in MIP-1α may simply be the result of an overall increase in the number of macrophages within the eye, rather than a change in the actual level of expression as a result of GM-CSF over expression. An answer to this and a determination of the precise cellular source, will have to await *in-situ* hybridisation studies.

Although, as discussed in section 6.11.4, the analysis of the GM-CSF transgenic bone marrow sample was inconclusive, the RT-PCR analysis did suggest that MIP-1α expression might be elevated in the GM-CSF transgenic bone marrow compared to the wild type. If this was indeed the case, then this would be somewhat unexpected, for the following reason. In the original characterisation of the GM-CSF transgenic mice reported by Lang *et al* (1987), no expression of the GM-CSF transgene was reported in the bone marrow of transgenic mice and therefore an increase in MIP-1α expression may be unexpected. It should be noted however, that this original analysis of transgene expression was characterised by Northern blot analysis rather than by the more sensitive technique of RT-PCR analysis and it is therefore possible that the GM-CSF transgene is indeed expressed at low levels in the transgenic mice. Furthermore, the serum levels of GM-CSF were reported to be elevated, by a much as 40-fold in GM-CSF transgenics (Lang *et al* 1987). This could provide a possible explanation, if increased MIP-1α expression does occur in transgenic bone marrow, relative to that of wild type bone marrow. Clearly the above discussion is merely speculative and can only be confirmed by repeating this analysis.

The observation that the level of MIP-1α expression was unaltered in the striated muscle of a transgenic mouse, as indicated in figures 6.30 and 6.31, is perhaps surprising in the light of observations made during the initial characterisation of GM-CSF transgenic mice by Lang *et al* 1987). In this, the authors reported that the expression of the transgene did not occur in non-infiltrated muscle of GM-CSF transgenic mice. Furthermore, this transgene expression was age dependent and was actually reported to follow, rather than precede the development of lesions in transgenic mice exhibiting infiltrates in their striated muscle. As a result of these observations, the authors proposed that macrophage accumulation was unlikely to be the direct cause of local GM-CSF expression and suggested that GM-CSF might

merely prolong the survival of macrophages already present within the striated muscle, thus leading to an indirect accumulation of macrophages. The results discussed in section 6.11.5 suggest that MIP-1α expression is not elevated in the striated muscle of GM-CSF transgenic mice. The reason for the discrepancy between this result and that expected is unclear. However, as noted previously in section 6.11.5, some genomic contamination was present in the GM-CSF transgenic samples analysed and this may have resulted in the sequestration of primers. If this is indeed the case then this might explain the absence of any elevated level of MIP-1α expression in striated muscle, particularly if the magnitude of any increase was relatively small.

GM-CSF transgenic mice show a very striking increase in the cellularity of their peritoneal cavities, with 90 % of these peritoneal cells being of an enlarged macrophage phenotype and showing characteristics of a functionally activated state. Given the potent effect of GM-CSF on macrophages as described during the course of this work and the reported expression of the GM-CSF transgene in the peritoneal cavity, one would perhaps expect to see an increased expression of MIP-1α in peritoneal cells derived from a transgenic mouse, compared to the level of expression observed in a littermate control. As seen in figures 6.32 and 6.33, this was apparently not the case. It should be noted that BMM were used throughout the course of the studies reported herein and these may be quite distinct in comparison to other macrophages, such as peritoneal macrophages (reviewed by Gordon 1995). Obviously, it would be necessary to first compare the effect of GM-CSF on BMM and peritoneal macrophage expression of MIP-1α, in order to answer this question. It is therefore theoretically possible that peritoneal macrophages may respond differently to GM-CSF, compared to resident BMM.

Whilst this examination of MIP-1α in GM-CSF transgenic mice is only preliminary, it does offer some tentative suggestion that MIP-1α expression may be elevated in specific tissues of transgenic mice, compared to wild type mice. It should however be noted that the results of such RT-PCR analysis should always be treated with caution, due to the extreme sensitivity of RT-PCR. These results do however, suggest that further examination of larger numbers of GM-CSF transgenic mice would be warranted, perhaps in conjunction with *in situ* hybridisation analysis.

7.8 The Role of GM-CSF, IL-3 and TGF-β in Regulating the Expression of MIP-1α in BMM

Previous work in our laboratory identified an endogenous reciprocal relationship between MIP-1α and TGF-β in BMM. The studies performed during the course of this thesis have added yet another layer of regulation to those previously identified. The results suggest, that in addition to the suppressive effects of TGF-β, stimulation of MIP-1α expression is mediated by GM-CSF and IL-3. Moreover, this inductive effect may be potent enough to overcome the suppressive block exerted on these cells by TGF-β. Taken together this implies that under the appropriate conditions, the expression of GM-CSF and IL-3 may be induced and this may be sufficient to overcome the TGF-β block on MIP-1α expression.

So are these observations pertinent to the regulation of MIP-1α during haemopoiesis? BMM are an excellent *in vitro* model cell system, roughly equivalent to the resident bone marrow macrophage population that is believed to be the primary source of MIP-1α in the bone marrow and therefore are ideally suited to the study of the regulation of MIP-1α expression. Interestingly, the levels of GM-CSF mRNA have been reported to increase in the bone marrow and spleens of mice, during the recovery period following sublethal irradiation (Chang *et al* 1995), whilst the levels of TGF-β were reported to remain constant. It would be of interest to determine if the level of MIP-1α expression was also increased, perhaps with a delayed kinetic response relative to that of GM-CSF expression? (see section 7.10). If the expression of MIP-1α was delayed with respect to that of GM-CSF, this may suggest that MIP-1α expression is induced via GM-CSF. If this were indeed the case, then one could speculate a role for MIP-1α in 'dampening down' the proliferation of haemopoietic stem cells following irradiation or cytotoxic drug treatment. One interesting approach would be to investigate this relationship in both normal and MIP-1α deficient mice and compare their recovery. Such investigation, coupled with *in-situ* hybridisation studies would also enable the precise cellular sources of this increased GM-CSF expression to be identified.

The previous study of Maltman *et al* (1996) suggested that the suppressive effect of TGF-β was relatively restricted to MIP-1α and MIP-1β. Studies reported in this work have suggested however, that this suppression is probably more widespread,

with TGF-β1 acting to suppress the GM-CSF/IL-3 induced expression of at least two additional chemokines, JE and MARC. Current evidence suggests that JE and MARC are not functional as inhibitors of stem cell proliferation and therefore the suppressive effect of TGF-β may be related to its widely documented role as an immuno-suppressive molecule. It is equally possible therefore, that the observed ability of GM-CSF and IL-3 to induce the expression of JE and MARC in addition to that of MIP-1α and MIP-1β, may also be more related to pro-inflammatory processes than to stem cell proliferative control. However this observation does not rule out the involvement of GM-CSF or IL-3, in stem cell proliferative control. As discussed in the introduction, GM-CSF, IL-3 and TGF-β are just three such factors and there are a multitude of additional growth factors, cytokines, receptors, adhesion molecules and other regulatory mechanisms, that could impinge upon their interactions. One must also consider the fact that these factors may function within highly localised niches in the bone marrow microenvironment, possibly through interaction with molecules such as proteoglycans that may act to sequester or even actively present growth factors and chemokines to target cells. Clearly, an *in vitro* culture system such as BMM can not provide a full re-creation of the complex environment of the bone marrow. Additional levels of control may also exist, such as through endogenous interactions between MIP-1α, GM-CSF and IL-3, and between TGF-β, GM-CSF and IL-3 and their receptors. Additionally, other cells besides macrophages are likely to be important in this co-ordinated regulation. The use of long-term bone marrow cultures, could provide a better model system in which to investigate the importance of these additional factors and cellular interactions.

The precise implications of the differences observed in the regulation of MIP-1α receptor expression remains unclear. No firm functional support could be observed for the observations made regarding the effect of GM-CSF at the level of mRNA expression, as determined by comparison of the ability to mobilise calcium in GM-CSF treated BMM. This may however, merely reflect technical problems. Indeed, the increase in CCR1 mRNA expression was reflected by an increase in the binding of MIP-1α to BMM, revealing that an increase in the cell surface expression of MIP-1α receptor numbers did occur in GM-CSF stimulated BMM. The potential effects of a 2-3 fold increase in receptor numbers on BMM function is unclear and warrants

further investigation. It is not possible to say with certainty whether the observed effects of GM-CSF and IL-3 on CCR1 expression relate to a proinflammatory effect or one involved in stem cell proliferative control, but several points suggest that it is more likely to be the result of a role in an inflammatory context. Firstly, the MIP-1α stem-cell inhibitory receptor has not yet been identified. Current evidence from our laboratory suggests that it is not CCR1 (Graham *et al* 1996), although others have suggested a role for human CCR1 in BFU-E inhibition (Su *et al* 1997). Secondly, the observation that CCR1 expression was also elevated in splenic cells, suggests that this pattern of expression may also occur in B and T cells (since B cells and to a lesser extent T cells, are a major cellular component of the murine spleen). A full understanding of the involvement of GM-CSF and IL-3 in the regulation of MIP-1α receptor expression, in the context of stem cell proliferative control, must therefore await identification of the receptor through which MIP-1α transduces the signal for stem cell inhibition.

So what is the function of GM-CSF, IL-3 and TGF-β in the regulation of MIP-1α expression? As discussed above, these studies have implicated GM-CSF and IL-3 in the regulatory control of MIP-1α expression in BMM. In the context of stem cell proliferative control, GM-CSF and IL-3 may function as stimulators of MIP-1α expression, whilst TGF-β may act to suppress this induction of MIP-1α. As discussed in sections 2.2 and 2.11, GM-CSF and IL-3 clearly function as haemopoietic regulators. So if GM-CSF and IL-3 upregulate MIP-1α expression, why does this not result in inhibition? One explanation maybe that full translation of this increased MIP-1α mRNA may not occur and indeed it is uncertain exactly what level of functional MIP-1α protein would be required to result in inhibition, in either autocrine or paracrine situations. In addition, it is always important to consider the many other factors that may be present in the bone marrow microenvironment. It is clear that MIP-1α functions within a relatively narrow window within the stem cell compartment and it may be speculated that MIP-1α acts as a stem cell inhibitor only during occasions of extreme stress, such as a systemic infection. During such an infection extensive haemopoietic stem cell proliferation may occur and one possible role for MIP-1α may therefore be to 'dampen down' proliferation of these cells.

7.9 The Importance of MIP-1α in Disease Pathology

The work presented in this thesis suggests that a complex relationship exists between MIP-1α and at least three other haemopoietic growth factors. This has important implications not just for the regulation of haemopoietic stem cell proliferation, but in the much wider context of a multitude of diseases and pathological states. Indeed it is now evident, particularly from the study of mice deficient in the functions of GM-CSF, IL-3 and IL-5, that GM-CSF and IL-3 may be important mediators of inflammation, as discussed in sections 2.2 and 2.11. For instance GM-CSF, IL-3 and MIP-1α have been implicated in the functions of mature eosinophil, basophil and mast cells. These cells are critical to the development of inflammatory disorders such as asthma and in the protection against parasitic infections. This complex picture is further complicated by the existence of over 35 other chemokines (see table 3.1), and others are likely to be identified in the future. Results presented in this thesis suggest that the relationship with GM-CSF and IL-3 appears relatively non-specific and can be extended to other chemokines. Indeed other researchers have shown that C10, MPIF-2 and IL-8 are all inducible by GM-CSF and/or IL-3 (Orlofsky *et al* 1994, Patel *et al* 1997, Takahashi *et al* 1993). Clearly this results in a very large and complex interacting network of inflammatory cytokines with potentially important pathological implications.

As discussed in section 3.6.3, MIP-1α is implicated in a wide variety of diseases and pathological states. Perhaps the most compelling evidence for the *in vivo* importance of MIP-1α is the report by Cook *et al* (1995). In this report, the authors generated mice in which the gene for MIP-1α was disrupted and demonstrated that these MIP-1$\alpha^{-/-}$ mice were resistant to coxsackievirus B3 (CVB3)-induced myocarditis and exhibited a reduced pneumonitis following influenza virus infection. The cardiac damage resulting from CVB3 infection therefore appears to be the result of the actions of recruited inflammatory cells, rather than from a direct effect of the virus. This report clearly demonstrated an *in vivo* role for MIP-1α in viral infection and has subsequently been followed by a number of other studies. For instance Epstein Barr virus (EBV) has recently been reported to induce the expression of MIP-1α (and IL-8) in human neutrophils, which may result in the chemoattraction of T and B cells and therefore enhance the ability of EBV to infect T and B cells (McColl *et al* 1997).

Infection of human monocytes with influenza A virus has been shown to result in the expression of MIP-1α and other CC chemokines (Sprenger *et al* 1996). A critical role for MIP-1α in NK cell inflammatory responses against murine CMV (MCMV) was also recently reported (Salazar-Mather *et al* 1998). These studies, combined with the mounting evidence from studies on HIV pathogenesis (see section 3.9) suggest that this role in viral pathogenesis is likely to be widespread. Indeed this importance is further underlined by the emergence of various virally encoded homologues of MIP-1α and chemokine-like receptors (see section 3.10)

MIP-1α is implicated in several autoimmune diseases, perhaps most notably that of MS. Indeed, administration of anti-MIP-1α antibodies results in prevention of experimentally induced MS (Karpus *et al* 1995). Intriguingly, TGF-β may also play a protective role in MS (Kuruvilla *et al* 1991) and given the clear relationship between TGF-β and MIP-1α, as detailed in this thesis, it is tempting to speculate that these may be linked. In a similar way, both GM-CSF and MIP-1α have been implicated in the development of rheumatoid arthritis (Koch *et al* 1994, Haworth *et al* 1991).

The suppressive actions of TGF-β may also reflect an involvement in anti-inflammatory processes and this suggestion is supported by the suppressive effect of TGF-β1 on other chemokines such as JE and MARC. The observation that MIP-1α expression is elevated in TGF-β1 null mice suggests that this suppressive role is likely to be important *in vivo*.

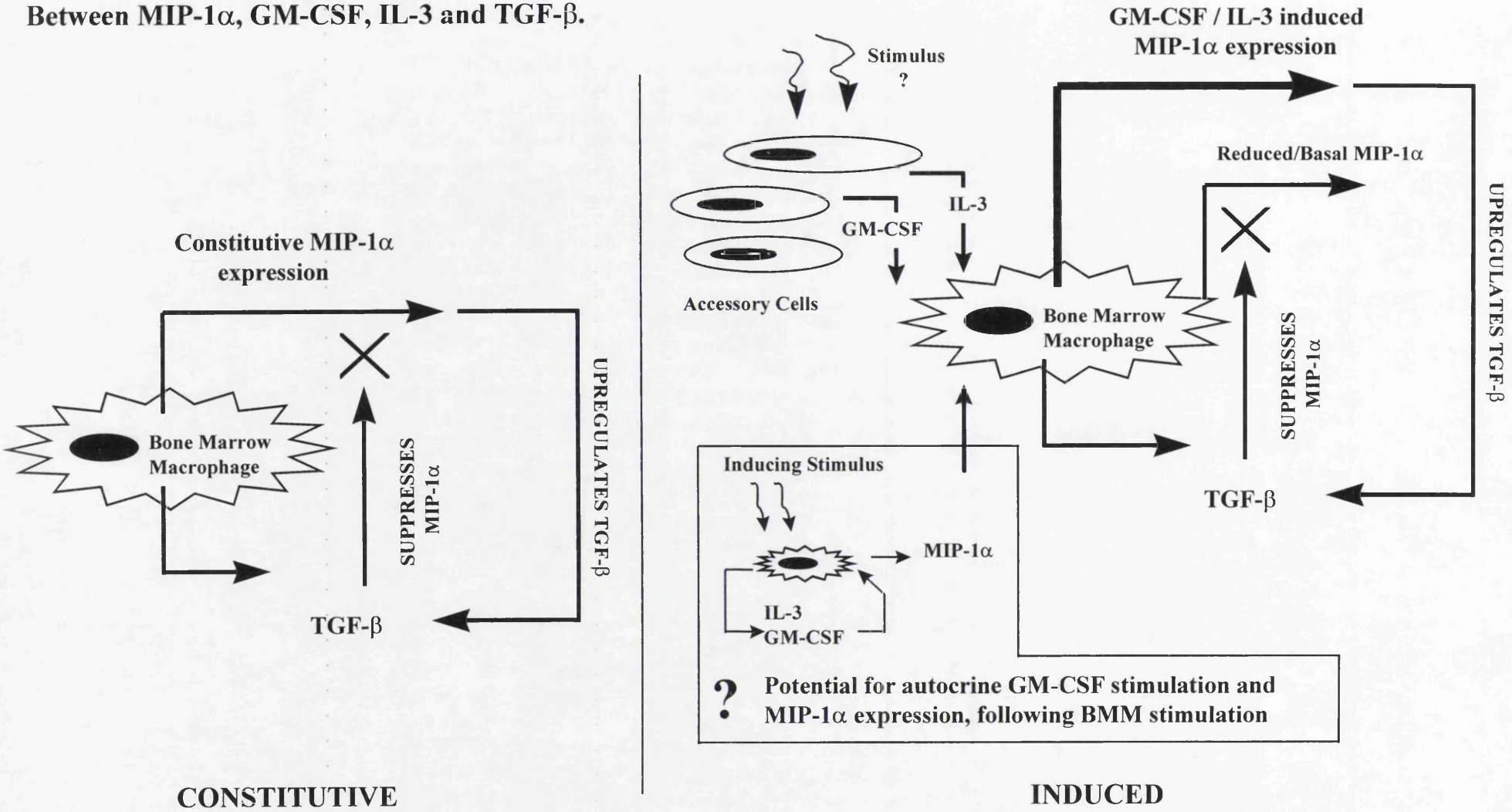
7.10 Conclusions and aspects for further investigation

MIP-1α, in addition to its roles in the normal function of the immunohaemopoietic system, is clearly implicated in a wide variety of diseases and is important in many pathological circumstances. This underlines the need to further understand how its expression is controlled under normal circumstances. The aim of this thesis was to attempt to further understand the network of regulatory factors controlling the expression of MIP-1α in the bone marrow. As discussed above, this included a previously identified suppressive role for TGF-β. With the observation that GM-CSF and IL-3 appear to function as stimulators of MIP-1α mRNA expression in BMM, yet further complexity is now evident in the existing network of factors involved in MIP-1α expression. In conclusion, both stimulatory and inhibitory

mechanisms appear to regulate the expression of MIP-1α and other chemokines in BMM, forming a complex interacting network between GM-CSF, IL-3, TGF-β, MIP-1α, other chemokines and perhaps even some of their receptors. Figure 7.1 provides a summary of the proposed interactions between MIP-1α, TGF-β, GM-CSF, IL-3 and other factors in BMM, as suggested by work reported in this thesis.

Many others aspects of these interactions remain in question. For instance, is there also a reciprocal relationship between MIP-1α and GM-CSF / IL-3 and what effect does TGF-β have on the expression of GM-CSF and IL-3? A previous report has suggested that TGF-β downregulates the expression of receptors for GM-CSF and IL-3 on murine haemopoietic progenitor cell lines (Jacobsen *et al* 1991a). If TGF-β is also able to suppress GM-CSF and IL-3 receptor expression on BMM, then this could result in a reduced responsiveness of BMM to GM-CSF and IL-3 (with a subsequent reduced expression of MIP-1α). TGF-β has also been reported to downregulates TNF-α or IL-1-induced GM-CSF expression in human articular chondrocytes (Alsalameh *et al* 1994). Further investigation is required to ascertain the precise relationship between these different factors and how this affects their individual expression, in both constitutive and induced circumstances. For instance, it is uncertain whether or not BMM constitutively express GM-CSF or IL-3. A study by Temeles *et al* (1993) suggests that BMM do not express mRNA for either GM-CSF or IL-3. This would imply that GM-CSF and IL-3 do not function in an autocrine manner to regulate MIP-1α expression in BMM. This observation is in agreement with experiments using neutralising antibodies against GM-CSF and IL-3, the results of which are shown in figures 6.6 and 6.8B respectively. This suggests that under uninduced conditions, BMM are not a source of GM-CSF or IL-3 and that they must be produced by other types of accessory cells within the bone marrow microenvironment. Although in the study reported by Temeles *et al* (1993), stimulation of BMM with cytokines did not result in the induction of GM-CSF or IL-3 expression, it is possible that induction of GM-CSF and IL-3 may occur in BMM following an appropriate inductive stimulus (such as LPS?). This induced GM-CSF and IL-3 could then operate in an autocrine manner to induce MIP-1α expression. This potential for 'induced autocrine' expression is also illustrated in figure 7.1. It is unlikely that this is the whole story, as other growth factors within the bone marrow microenvironment are almost certain to

Figure 7.1: Summary of Proposed Interactions Between MIP-1α, GM-CSF, IL-3 and TGF-β.



affect the expression of GM-CSF, IL-3, TGF-β and MIP-1α. For example, IL-1, TNF-α and IFN-α have all been reported to affect GM-CSF expression in a variety of cell types (Fibbe *et al* 1986, Gollner *et al* 1995). This creates the potential for yet further complexity in this network.

A molecular analysis of the precise mechanisms, such as transcription factors and other downstream signal transduction pathways, through which GM-CSF and IL-3 achieve induction of MIP-1α expression, is also worthy of further investigation. As discussed earlier in section 7.4, such an analysis could prove particularly informative, if coupled with analysis of other chemokine genes, such as JE/MCP-1. Preliminary results, not presented in this thesis, obtained from studies using the protein synthesis inhibitor Cycloheximide have indicated that GM-CSF-induced MIP-1α expression is at least in part transcriptionally regulated. Analysis of the molecular elements involved in the regulatory control of MIP-1α expression by GM-CSF and IL-3 is clearly an area with extensive potential for further investigation. This could also be coupled with an analysis of those pathways involved in TGF-β1 mediated suppression of MIP-1α expression, with the aim of determining exactly how TGF-β suppresses MIP-1α expression. In fact, a recent report has suggested that TGF-β suppreses MCP-1 expression in macrophages, possibly by downregulating the expression of c-jun, one of the components of the AP-1 transcription factor complex (Kitamura *et al* 1997). It would be of interest to examine this observation further in the light of the results presented here in this thesis and this might provide further clues as to why GM-CSF, IL-3 and TGF-β appear to be so non-specific in their effects on chemokines.

One final area worthy of further attention, is an investigation into the *in vivo* interactions between MIP-1α, GM-CSF, IL-3 and TGF-β1. It is assumed that the regeneration of the haemopoietic system after sublethal myeloablation involves the action of endogenously produced haemopoietic growth factors. Indeed, following *in vitro* irradiation the levels of GM-CSF protein were previously reported to increase in murine long-term bone marrow cultures and spleen cell cultures (Naparstek *et al* 1985, Onoda *et al* 1980). A study reported by Chang *et al* (1995), demonstrated that a marked increase in GM-CSF mRNA occurred during the first two weeks of the recovery period following sublethal irradiation. This increase in GM-CSF mRNA expression appeared to precede the cellular recovery, which did not appear until

approximately 2 weeks post-irradiation. Therefore, an analysis of MIP-1α expression, together with that of GM-CSF, IL-3 and TGF-β, in sublethally irradiated mice could prove highly informative. Admittedly such an analysis might however be relatively difficult to undertake, given the relatively low levels of cytokines present in bone marrow and particularly with the very small numbers of cells that would be present during the earlier stages of reconstitution of the bone marrow microenvironment.

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