Investigation of MIP-1α inhibitory activity on the CFU-A stem cells

by

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Declaration

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

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Abstract

Macrophage inflammatory protein-1alpha (MIP-1α) is a member of the chemokine superfamily and has been observed to inhibit the proliferation of transiently engrafting stem cells, namely the colony forming unit-agar (CFU-A) stem cells. This study was initiated to investigate how MIP-1α exerts its inhibitory effect on these cells at the genetic level and, to examine whether altering the growth factors required for CFU-A colony formation could interfere with the inhibitory activity of MIP-1α.

The results presented in this thesis indicate that growth factor alteration has minimal effects on the inhibition of CFU-A colony formation. However, low levels of MIP-1α in the presence of high levels of SCF or M-CSF have been observed to stimulate colony formation. This stimulatory activity of MIP-1α has been previously observed on progenitor cells however, it has never been reported on stem cells. Furthermore, alternatively shaped CFU-A colonies have been observed in assays containing high levels of both GM-CSF and MIP-1α. These results indicate that although the growth factors in the context of this assay can not interfere with the inhibitory signal of MIP-1α they may however interact with the other MIP-1α signalling pathways.

Although in the CFU-A assay SCF and IL-11 could not interfere with the inhibition of CFU-A colonies by MIP-1α, it was observed that upon the ex-vivo expansion of bone marrow, with SCF and IL-11, that the inhibitory activity of MIP-1α was reduced. This effect was observed to be specific for MIP-1α, as TGF-β inhibition of CFU-A colony formation was not affected, and was proposed to be due to the down regulation of the MIP-1α inhibitory receptor. Indeed analysis of MIP-1α receptor expression indicated that CCR-1 was up-regulated whereas CCR3 and D6 were both down regulated, however neither CCR-3 nor D6 proved to be involved in MIP-1α inhibition of CFU-A colony formation. Therefore this study observed that in the context of the CFU-A assay that MIP-1α inhibitory signalling pathway is robust and minimally interacts with SCF, M-CSF, GM-CSF, IL-11 and LIF signalling pathways.

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ABBREVIATIONS

Ac	acetyl
ACE	angiotensin converting enzyme
AIDS	acquired immune deficiency syndrome
aa	amino acid
Ara-C	cytosine arabinoside
βc	common β chain receptor subunit
bcl	b cell lymphoma
BFU-E	burst Colony forming unit erythroid
BM	bone marrow
BMM	bone marrow macrophage
BSA	bovine serum albumin
CAM	cellular adhesion molecule
C	cysteine
CCE	centrifugal counterflow elutriation
cDNA	complementary deoxyribonucleic acid
CFC	colony forming cell
c-fms	M-CSF receptor
CFU	colony forming unit
CFU-A	colony forming unit agar
CFU-E	colony forming unit erythroid
CFU-G	colony forming unit granulocyte
CFU-GEMM	colony forming unit granulocyte/ erythroid megakaryocyte /macrophage
CFU-GM	colony forming unit granulocyte/ macrophage
CFU-M	colony forming unit macrophage
CFU-Meg	colony forming unit megakaryocyte
CFU-S	colony forming unit spleen
c-kit	stem cell factor receptor
CLP	common lymphoid progenitor
CM	conditioned media
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CNS	central nervous system
CNTF	ciliary neurotropic factor
CSF	colony stimulating factor
CT	cardiotropin
CTP	cytidine triphosphate
D	Aspartic acid
d	day (e.g day 12)
DARC	Duffy antigen receptor for chemokines
dATP	deoxyadenosine triphospahte
DEPC	diethylpyrocarbonate

dCTP	deoxycytidine triphosphate
dGTP	deoxyguanine triphosphate
DHS	donor horse serum
DMEM	Dulbecco's modified eagles medium
DMSO	dimethylsulphoxide
DNA	deooxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuracil triphosphate
E	Glutamic acid
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ENA	epithelial neutrophil attractant
EPO	erythropoietin
ES	embryonic stem
est	expressed sequence tags
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDCP	factor dependant cell Paterson
flk	fetal liver kinase
flt	fms like tyrosine kinase
FL	Flk/flt ligand
FU	fluorouracil
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCP	granulocyte chemotactic protein
G-CSF	granulocyte colony stimulating factor
GM	granulocyte /macrophage
GM-CSF	granulocyte macrophage colony stimulating factor
GMP	granulocyte / macrophage progenitor
GPCR	g-protein coupled receptor
gp	glycoprotein
GRO	granulocytes related oncogene
GTP	guanosine triphosphate
h	human
HIV	human immunodeficiency virus
HPC	haemopoietic progenitor cell
HPP-CFC	high proliferative potential colony forming cell
HS	heparan sulphate
HSC	haemopoietic stem cell
HU	hydroxyurea

IFN	interferon
Ig	immunoglobulin (e.g. IgM)
IGF	insulin like growth factor
IL-	interleukin (e.g IL-6)
IP10	interferon-inducible protein-10
IP3	inositol triphosphate
JAK	janus activated kinase
K	lysine
kDa	kilodaltons
KSHV	kaposi's sarcoma-associated herpesvirus
L	leucine
LB	Luria broth
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LTRC	long term repopulating cell
LTC-IC	long term culture initiating cell
LTBMC	long term bone marrow culture
Ltn	lymphotactin
m	murine
M-CSF	macrophage colony stimulating factor
MCP	monocyte chemotactic protein
MEM	minimal essential medium
MEP	megakaryocyte /erythrocyte progenitor
Mig	monokine induced by γ-interferon
MIP	macrophage inflammatory protein
MPIF	myeloid progenitor inhibitory factor
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
m.w	molecular weight
NK	natural killer
OD	optical density
op	osteopetrotic
OSM	oncostatin M
P	proline
PAP	pulmonary alveolar proteinosis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PF-4	platelet factor-4
PG	proteoglycan
PHSC	pluripotent haemopoietic stem cell
PKC	protein kinase C

PLC	phospholipase C
PTX	pertussis toxin
r	recombinant
R	receptor or Arginine
RA	rheumatoid arthritis
RANTES	regulated on activation normal T-cell expressed and secreted
Rh	rhodamine
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
RTK	receptor tyrosine kinase
S	serine
s	soluble
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCI	stem cell inhibitor
SCM	single cysteine motif
SDF	stromal derived factor
SH2	src homolgy region 2
Sl	steel
SOC	super optimal catabolite
STAT	signal transducer activated transcription
T	Threonine
tg	transgenic
TGF	transforming growth factor
TNF	tumour necrosis factor
TPO	thrombopoietin
U	units
UTP	uridine triphosphate
VLA	vascular leukocyte antigen
v/v	volume for volume
W	white locus or tryptophan
WGA	wheat germ agglutinin
w/v	weight for volume
wt	wild type

CHAPTER 1 : INTRODUCTION

1.1 The Haemopoietic System : An Overview

The haemopoietic system is a complex cellular system, which is essential for the maintained viability of an individual. It consists of at least eight, phenotypically and functionally distinct mature cells with an overlapping set of more immature cell types (figure 1.1). All immature cells and mature blood cells are ultimately derived from the self-renewing pluripotent stem cell through the processes of commitment, proliferation and differentiation. The mature blood cells have well characterised morphologies and functional properties. Indeed, erythrocytes are responsible for oxygen and carbon dioxide transport, megakaryocytes generate platelets necessary for clotting the blood; T and B cells contribute to immune surveillance and responsiveness; and macrophages and granulocytes aid in the disposal of invading micro-organisms and damaged tissue. A necessary property of the haemopoietic system is the capacity to regenerate mature cells and therefore maintain steady state levels. It has been estimated that to maintain homeostasis in humans, the haemopoietic system must replace approximately 2.4 $x10^8$ red blood cells and 4 $x10^6$ neutrophilic lymphocytes cells each day (Erslev 1983, Dancey *et al* 1976). Such demands necessitate strict control over haemopoietic progenitor proliferation and this is regulated by the various factors produced in the bone marrow microenvironment (section 1.7). These PHSC cells reside within the bone marrow and are defined as being able to self renew and give rise to all lineages of blood cells (Till and McCulloch 1960). This definition can be applied to a number of cells within the haemopoietic system, thus allowing one to refer to the primitive end of the system as the stem cell compartment (section 1.2 and figure 1.2). Stem cells only make up a tiny proportion of the cells in the haemopoietic system, probably between 0.01-0.1 % of the total bone marrow and the majority of these stem cells are quiescent (Hodgson *et al* 1982). Under steady state conditions, the quiescent stem cells play a minimal role in haemopoiesis and constitutive haemopoiesis is thought to be maintained by the more mature stem and progenitor cells. However, under haematological stresses such as blood loss, infection and exposure to cytotoxic chemicals, the stem cells can be rapidly induced into cell cycle to replenish the mature cell compartment and this is

Figure 1.1 Overview of Haemopoiesis

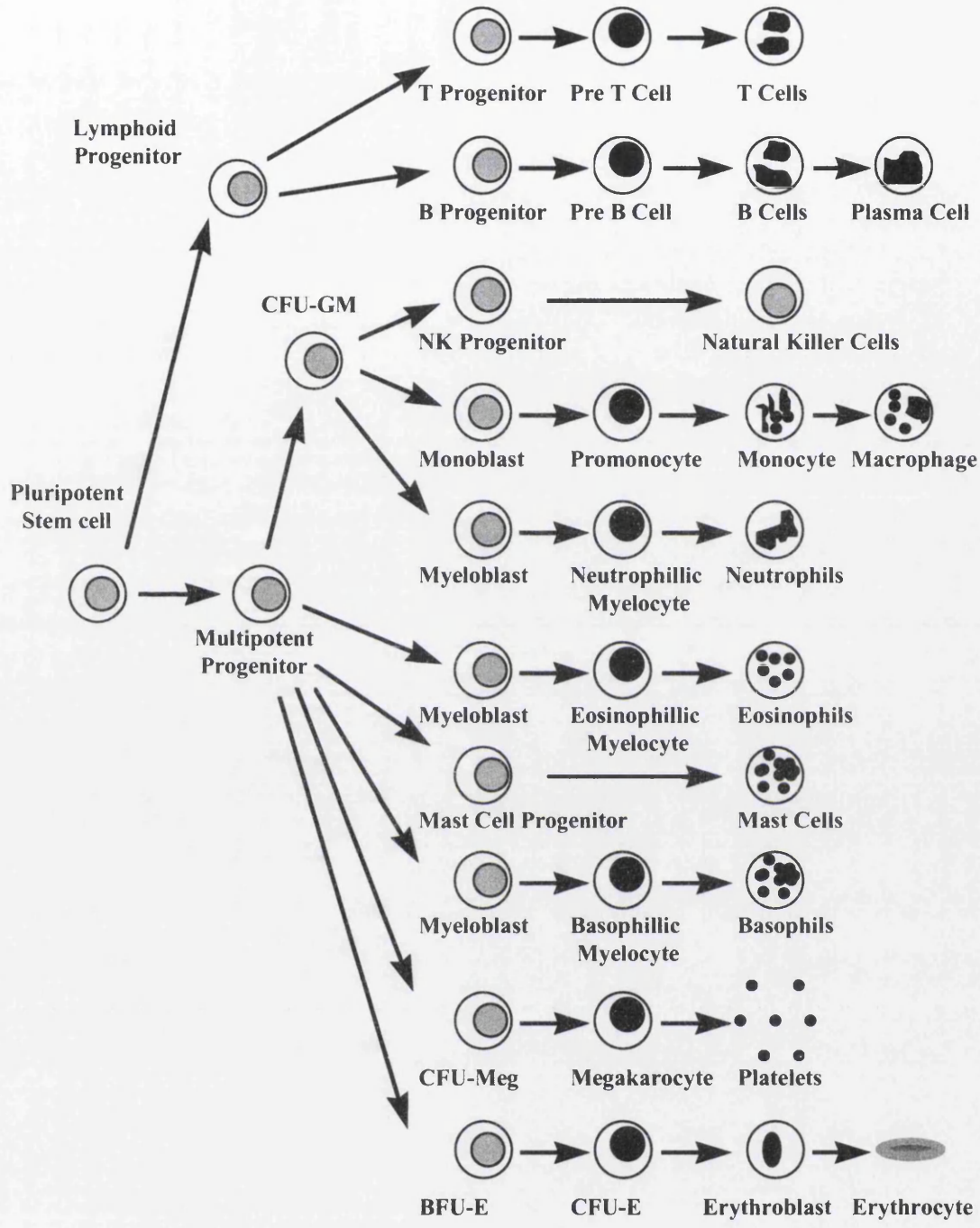


Fig 1.1 Overview of haemopoiesis

A schematic representation of the haemopoietic system, depicting a few of the precursor cells that are involved in the production of the mature blood cells. CFU-GM Colony Forming Unit-Granulocyte macrophage, BFU-E Blast Forming Unit-Erythroid, CFU-E Colony Forming Unit-Erythroid, CFU-Meg Colony Forming Unit Megakaryocyte, NK Natural Killer .

known as inducible haemopoiesis. Following the replenishment of the system, these stem cells once again become quiescent.

These observations suggest that the HSC is under both positive and negative proliferative regulation and that the overall proliferative state of the HSC is dependent upon the levels of these two opposing activities (sections 1.7 and 1.9).

1.2 The Stem Cell Compartment

The variation in cell cycling properties, and self renewal capacity of the CFU-S stem cells, lead Rosendaal *et al* (Rosendaal *et al* 1976) to suggest that the stem cell compartment is organised in a hierarchical structure. Young stem cells that have undergone few divisions having extensive self-renewal capacity and a lower differentiation drive are near the primitive end of the system, whereas, the older stem cells that are more mature, have the opposite properties of the young stem cells in that they are limited in their self-renewal capacity, and have a greater tendency to differentiate.

Using various *in-vivo* and *in-vitro* assays, it has been demonstrated that at the primitive end of the haemopoietic system are a range of overlapping cell types that display the characteristics of stem cells, but differ in their relative abilities to self renew and differentiate. These findings have lead to the definition of the primitive end of the system as the stem cell compartment and one can imagine the stem cell compartment represented as a pyramidal structure (figure 1.2). At the top of the triangle are the most primitive cells, the pluripotent haemopoietic stem cells, these cells display a high self-renewal capacity, repopulating ability and are most resistant to differentiation and proliferation stimuli. As one goes further down the stem cell compartment, the cells display a reduced tendency to self-renewal with a concurrent increase in capacity to differentiate, and the mature blood cells are found at the base of the pyramid. Therefore, the stem cell compartment is a heterogeneous compartment consisting of cells displaying varying degrees of self-renewal capacity and/ or differentiation potential (Graham *et al* 1992a).

Figure 1.2 : The Stem Cell Compartment

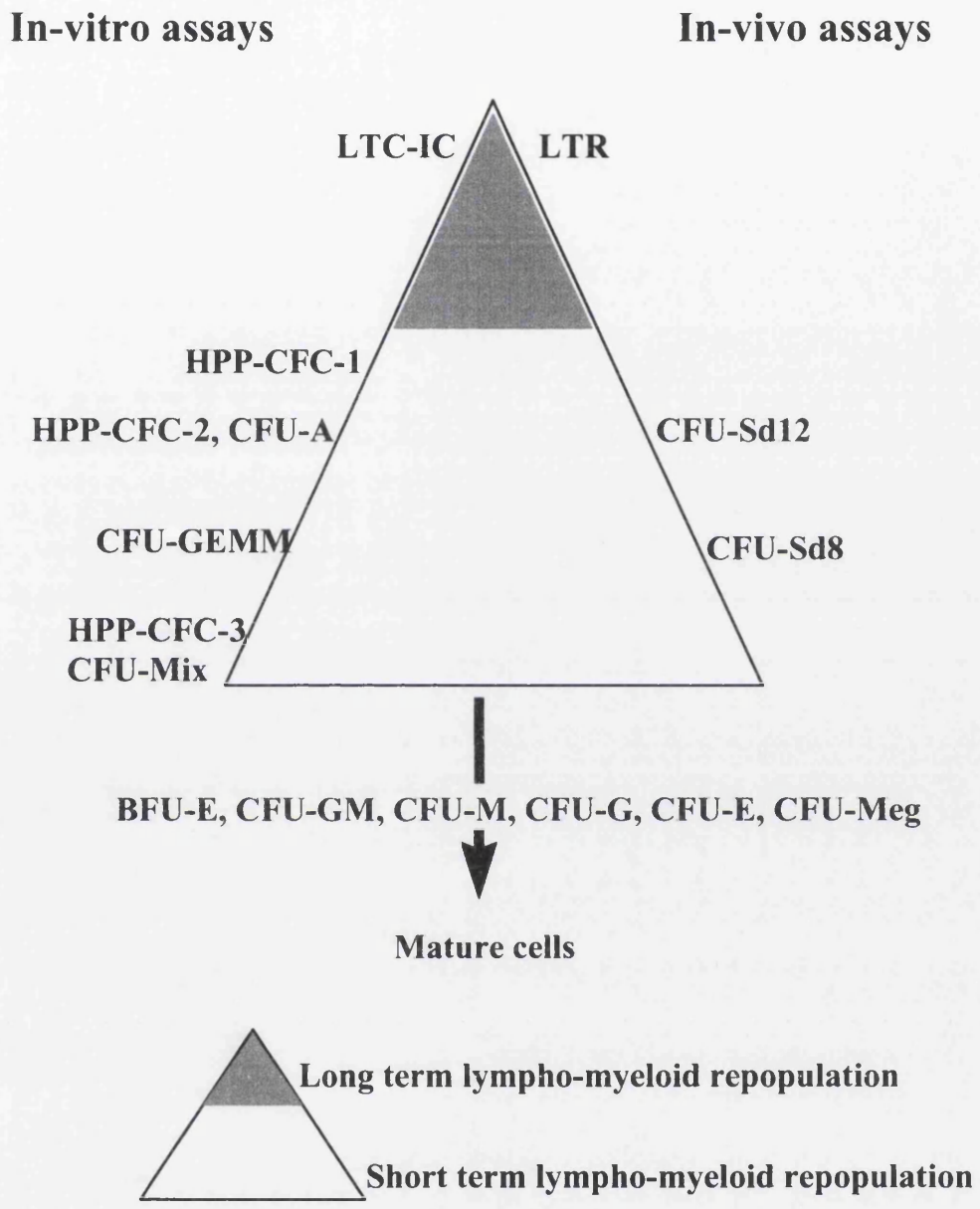


Fig 2.1 The Stem Cell Compartment

A schematic representation of the stem cell compartment. The LTC-IC and LTR have the greatest self-renewal and long term repopulating abilities, whereas the stem cells downstream of these have more proliferative potential and have lower self-renewal capacities. The progenitor assays depicted under the triangle also have high proliferative capacity and give rise to mature cell colonies in agar or methylcellulose, from where they get their name. The traingle is split into two parts, which define the long (grey triangle) and short term repopulating potential of these parts of the stem cell compartment, both of which are needed for long term survival after marrow ablation by chemotherapy.

The stem cell compartment can be further simplified within the murine system based on the abilities of the members of the stem cell compartment to confer engraftment of the haemopoietic system after lethal irradiation. Lethal irradiation of a mouse will ensure death as a result of the ablation of the haemopoietic system due to the attendant bone marrow failure. However, it is possible to rescue these irradiated mice by giving them a bone marrow transplant, during which the transplanted bone marrow cells replenish the damaged system, allowing the mouse to survive.

The stem cell compartment can be divided into 1) very primitive stem cells or long term repopulating cells and 2) less primitive stem cells or short term repopulating cells, that display distinct functional roles following transplantation (figure 1.2). Following irradiation of a mouse, the administration of only the less primitive stem cells rescue the mouse from radioactive insult, however, the mouse will die after 4-6 weeks from bone marrow failure. This indicates that the less primitive stem cells are only capable of transiently engrafting the murine haemopoietic system and can only protect against the initial radioactive insult. These cells are known as the transient engrafting cells, short term repopulating cells or radio-protective stem cells and typically have only limited differentiation potential (Jones *et al* 1990). In contrast, the administration of the very primitive stem cells following irradiation fails to rescue the mouse. The very primitive stem cells have no short term repopulating ability and do not protect the mouse against the radioactive insult. Therefore, to rescue a mouse and allow for haematological recovery, the combination of these two populations is needed. The less primitive stem cells act initially to allow the mouse to recover from the radioactive insult and during this time the very primitive cells seed within the bone marrow, and after a period of 4 to 6 weeks start to contribute to the haemopoietic system of the transplanted mouse. Thus the long-term repopulating stem cells allow the mouse to survive long term and as they are pluripotent, they are capable of generating both lymphoid and myeloid cells (Jones *et al* 1990, Uchida *et al* 1994).

1.3 Isolating Haemopoietic Stem Cell

There are numerous separation techniques used to enrich for haemopoietic stem cells, for example, stem cells can be enriched by their physical properties using density gradient centrifugation. This was demonstrated by Boyum *et al* (Boyum 1968) who

produced a one step centrifugal technique for isolation of various leukocytes (LymphoPrep^{TM}), this was modified in 1983 to isolate mononuclear cells from species other than humans. Both LymphoPrep^{TM} and NycoPrep^{TM} remove erythrocytes by sedimentation and the majority of mononuclear cells can be found separated at the interface of the plasma layer and the NycoPrep^{TM} solution (Boyum 1983). Counter flow centrifugal elutriation (CCE) is another centrifugation method used for separating different cellular populations and separates large numbers of cells, primarily on the basis of size and density. The cells are placed in a rotating chamber, and as they reach equilibrium, the smaller cells migrate to the centre of the chamber while the larger ones sediment towards the outer end and, with alterations in flow rate of the elutriation, cells of different sizes can be collected (Jones *et al* 1990).

Alternatively, the isolation of stem cells can be performed by examining the cell surface expression of various markers, such as lectins or cellular antigens via antibodies conjugated to fluorescent markers or, by the incorporation of vital dyes such as Rhodamine 123 or DNA binding dyes such as, Hoechst 33342. The basis of fluorescence activated cell sorting (FACS), is to label cells with one or a number of fluorescent markers and to use these to select a specific cellular population. Using a flow cytometer, this is performed on the basis of size and granularity and the heterogeneity of the fluorescent intensity of the population. Although there are no monoclonal antibodies available yet that exclusively recognise HSCs, these cells have been enriched by capitalising on multiple cell surface characteristics such as expression of *c-kit* (Okada *et al* 1991), Thy-1 (Muller-Seiburg *et al* 1986), Sca-1 (Spangrude *et al* 1988), CD34 (Sutherland *et al* 1992) or binding to wheat germ agglutinin (WGA) (Ploemacher *et al* 1988).

Although the use of various antibodies to surface antigens has allowed the separation of cellular populations that display stem cell characteristics, the relevant functional role of the expression of some of these antigens on PHSC is not so obvious. In an attempt to examine the function of these antigens, several groups have produced mice that do not express these various antigens.

Sca-1 or Ly-6A is expressed on most peripheral lymphocytes, as well as haemopoietic stem and progenitor cells (Shevach *et al* 1989), and surprisingly, analysis of Sca-1 null mice by flow cytometer demonstrated that these animals had normal levels of all

haemopoietic lineages in bone marrow, spleen, lymph node, and thymus, thereby demonstrating that Sca-1 is not necessary for haemopoietic development. However, it was observed that the T cells from these animals proliferate at an increased level compared to that of wild type cells, thereby suggesting that Sca-1 may be essential for regulation of T cell proliferation (Stanford *et al* 1997).

CD34, another supposed marker of PHSC activity, has been observed to be expressed on a heterogeneous population of haemopoietic cells which include primitive haemopoietic stem cells and committed progenitors of the myeloid, lymphoid and erythroid lineages (Sutherland *et al* 1992). The analysis of the function of CD34 *in-vivo* was performed by generating CD34 null mice by homologous recombination. These mice were observed to have a delay in myeloid and erythroid development and a reduced colony forming ability of their progenitor cells from the yolk sac and fetal liver. Furthermore, the colony forming potential of the haemopoietic progenitors from these mice (CFU-GM, CFU-GEMM and BFU-E) is reduced and these progenitor cells could not be expanded by the combination of SCF, FL, EPO and GM-CSF treatment however, in spite of these haemopoietic abnormalities the adult mice still display normal levels of mature blood cells. These results suggest that CD34 is involved in the proliferation and the maintenance of haemopoietic progenitor cells in the embryo and the adult mouse (Cheng *et al* 1996), however, the normal levels of mature blood cells in CD34 null mice suggest that CD34 may not be essential for the development of all haemopoietic lineages. Several other reports have observed PHSC activity in a $CD34^-$ cellular population, and upon stimulation of this $CD34^-$ population with growth factors, these cells become $CD34^+$, suggesting that CD34 may be a marker of activation in these PHSCs (Osawa *et al* 1996, Morel *et al* 1998).

Naturally occurring mutations in mice resulting in the lack of the stem cell factor SCF receptor (*c-kit*) have rendered the production of *c-kit* null mice by homologous recombination unnecessary. The absence of *c-kit* in these mice, results in death in utero as a result of severe anaemia and analysis of non-lethal mutations within the *c-kit* or *W* locus have indicated a further role for *c-kit* in the fertility and pigmentation of these mutant mice. On further examination of the bone marrow of W/W^v mice (Russell 1979), it was observed that these mice had fewer colony forming units spleen (CFU-S), burst forming unit erythroid (BFU-E), colony forming unit granulocyte macrophage (CFU-GM), and colony forming unit erythroid (CFU-E) than their litter

mate controls (Barker 1994). The reduction in progenitor cells and the lethal anaemia in the various *c-kit* mutant mice, indicate that *c-kit* as well as being a marker for PHSC may also play an important role in the survival and regulation of various haemopoietic cells and therefore, may be a more functionally relevant marker of PHSC than CD34 or Sca-1. However, more recent studies in the human and murine systems have observed that PHSC activity can also be isolated in a cellular population that is c-kit $^-$ (Sogo *et al* 1997, Doi *et al* 1997, Ortiz *et al* 1999). Therefore, although Sca-1, CD 34 and c-kit have been historically accepted as markers for isolating PHSC activity, it may be that their expression is not needed for the functional activity of these cells. It may even be possible that a definitive marker for the PHSC does not exist. However, following the intravenous introduction of haemopoietic stem cells, they find their way to the bone marrow with great accuracy. Therefore, it seems that these HSC carry certain cell surface molecules that are specific for counter receptors/ligands within the bone marrow stroma, thus indicating that there may be an as yet, unknown surface molecule that could be used as a marker for PHSC.

1.4 In-vitro Assays

Due to the difficulty in distinguishing stem cells on the basis of their morphology, much of what we know about stem cells has been learnt through the development and use of *in-vitro* assays. The basis of these *in-vitro* 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 combinations of cytokines and their concentrations vary from assay to assay, as does the size, morphology and cellular composition of these colonies, and these colony characteristics can be used to give an indication of the level of primitiveness of the cell from which they were derived. A number of *in-vitro* assays have been reported that detect a range of cells within the haemopoietic stem cell compartment. However, the precise relationship between these cells and the roles they 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 regulatory factors, a number of which are described in sections 1.41, 1.42 and 1.43 also see figure 1.2.

1.4.1 High Proliferative Potential-Colony Forming Cells (HPP-CFC) Assays

In 1979, Bradley and Hodgson described an *in-vitro* clonogenic assay, which detected a primitive stem/progenitor cell population termed, High Proliferative Potential Colony Forming Cells (HPP-CFC). These HPP-CFC cells form large macrophage colonies in agar culture that have diameters greater than 0.5 mm and contain somewhere in the region of 50000 cells per colony. The formation of HPP-CFC colonies was initially observed to be produced by synergistic interactions between M-CSF and crude conditioned media (CM) sources of other factors (Bradley and Hodgson 1979). However, with the use of recombinant growth factors, HPP-CFC stem cells could be subdivided into HPP-CFC-1, 2 and 3 (McNiece *et al* 1986). HPP-CFC-1 is the most primitive HPP-CFC sub-population, as indicated by their quiescence, their resistance to 5-FU and their ability to generate HPP-CFC-2 sub-population upon stimulation with SCF and M-CSF (McNiece *et al* 1986). These HPP-CFC-1 cells correlate closely with pre-CFU-S cells (Hodgson and Bradley 1984) and have been reported to generate CFU-S d12, cells of the megakaryocyte and granulocyte/macrophage lineages (McNiece *et al* 1987). Unlike HPP-CFC-1, that require IL-1, M-CSF and IL-3 for growth (McNiece *et al* 1987, HPP-CFC-2 and 3, colonies can be produced upon stimulation with only IL-3 / M-CSF and M-CSF respectively. Furthermore, HPP-CFC-2 and 3 numbers are depleted with 5-FU treatment, indicating that they are more mature and less quiescent than HPP-CFC-1 stem cells (McNiece *et al* 1987, 1988).

1.4.2 Colony Forming Unit-Agar (CFU-A) Assay

In 1988, Pragnell *et al* first described an *in-vitro* clonogenic assay that detects a transiently engrafting cell with similar characteristics to the CFU-S day 12 (Pragnell *et al* 1988). In this assay, bone marrow cells were grown in agar and medium supplemented with horse serum and a source of synergistic growth factors. These growth factors were, initially in the form of conditioned media (CM) from L-929 cells and AF-19T cells that produced M-CSF and GM-CSF respectively. The assay was further refined using recombinant growth factors, alongside M-CSF and GM-CSF, a further inclusion of SCF was required to produce similar numbers of CFU-A colonies seen with CM. This assay is incubated for 11 days, and under these conditions

macroscopic colonies of between 2-5 mm in diameter are produced, each of which contained on average 1-4 $x10^4$ cells. The incidence of CFU-A stem cells in normal bone marrow is 150-220/10^5 cells, although this varies slightly depending on the strain of mice (Lorimore *et al* 1990). Lorimore and colleagues characterised the CFU-A cell by comparing its properties with those of multipotential CFU-S d12 cells and the lineage restricted progenitor cells, GM-CFC. They observed that CFU-A and CFU-Sd12 cells displayed an identical recovery profile after a single dose of 5-FU, and that CFU-A and CFU-S cells derived from bone marrow were found to be out of cycle (<10% in S phase), and those derived from regenerating bone marrow were actively cycling (30% in S phase). Both CFU-A and CFU-Sd12 cellular populations responded identically when they were exposed to a proliferation inhibitor or stimulator and also displayed similar responses to ionising radiation. The similarity of CFU-A and CFU-Sd12 was further evident in experiments examining the cell separation of these cells on the basis of their density distributions and of their radial distribution in the femur (Lorimore *et al* 1990). Thus, the murine CFU-A and CFU-S d12 were shown to detect a similar transiently engrafting stem cell within the stem cell compartment, and interestingly, the CFU-A assay has also been reported to detect cells that can be detected by the more mature HPP-CFC assays. The CFU-A assay has been useful in the investigation into growth stimulators and inhibitors, indeed Graham *et al* used this assay to isolate and characterise the stem cell inhibitor, MIP-1α, and is routinely used in our laboratory (Graham *et al* 1990).

1.4.3 Long Term Culture Initiating Cells (LTC-IC)

In human and murine long-term bone marrow cultures, it has been observed that clonogenic progenitors rapidly undergo terminal differentiation (Sutherland and Eaves 1993, Kerk *et al* 1985). As a result, the initially present primitive progenitors disappear within the first 4 weeks, and the clonogenic progenitors detected at later times represent the progeny of more primitive precursors cells called long term culture initiating cells (LTC-IC). LTC-IC are present in normal bone marrow at a frequency of about 1/ $2x10^4$ nucleated cells and represent the most primitive stem cell detected *in-vitro*. A unique feature of the LTC, is its ability to better support the maintenance and proliferation of haemopoietic cells with stem cell characteristics, than standard colony assays. This is not only due to the production of growth factors needed for the extended haemopoiesis which is characteristic feature of LTCs, but

also to the supportive function provided by the feeder layer. Irradiated adherent layers from LTC, established with previous samples of normal (allogenic) bone marrow cells or alternatively murine fibroblast cell lines, can be used as a source of feeder layers in LTC. Human LTC-IC have a limited life span of approximately 2-5 weeks in long term bone marrow culture, however, a more recent study by Verfaillie observed that diffusable stromal factors from a stromal non contact assay, and the addition of MIP-1α and IL-3, allowed these human LTC-IC cells to survive for up to 2 months (Verfaillie *et al* 1995).

1.5 In-vitro Progenitor Assays

Progenitor cells are more lineage restricted cells and are capable of forming colonies that contain single or multiple cell types, and are named according to the type of cell that they give rise to in *in-vitro* colonies. Within the myeloid lineage, there are a number of progenitor cell assays that recognise the progeny produced by various progenitor cells in semi-solid assays. These include granulocyte, erythrocyte, megakaryocyte, macrophage colony forming unit (CFU-GEMM), granulocyte/ macrophage colony forming unit (CFU-GM), macrophage CFU (CFU-M), megakaryocyte CFU (CFU-Meg), the burst forming unit erythroid (BFU-E). A more recent study by Akashi *et al*, for the first time, described a method for isolating multipotential progenitors and committed progenitors from long term and short term repopulating stem cells. Indeed, they isolated a common myeloid progenitor (CMP) that can mature into two further committed progenitors, namely a granulocyte/ macrophage progenitor (GMP) and a megakaryocyte/ erythrocyte progenitor (MEP). These committed progenitors can terminally differentiate and produce granulocytes, macrophages, megakaryocytes and erythrocytes respectively (Akashi *et al* 2000). This study may lead to the further identification of the progenitor cells, such as BFU-E and CFU-M, whose existence has only been known due to their ability to produce colonies in semi-solid media, and to a better understanding of lineage commitment and the role each of these progenitors plays in haemopoiesis.

1.6 Bone Marrow Microenvironment

Mechanisms that govern induction of the quiescent state, proliferation and differentiation of primitive haemopoietic progenitors are not well understood. *In-vivo*

haemopoiesis takes place in close proximity with the bone marrow microenvironment where haemopoietic stem cells reside. This process is mimicked relatively closely *in-vitro* by the stroma-dependant long term bone marrow cultures (LTBMC), which were initially described by Dexter *et al* (Dexter *et al* 1977), and for human cells by Mergenthaler and Dormer (Mergenthaler and Dormer 1990). Initial evidence suggested that stem cells and progenitors bind to the stroma, whereas more mature cells are found as non-adherent cells in the supernatant (Coulombel *et al* 1983). However, Verfaillie indicated that although contact between HSC and stromal was observed to be initially needed, this dependency on contact can be replaced by diffusable soluble stromal factors and the addition of growth factors in a stroma non contact assay (Verfaillie *et al* 1994).

The long-term bone marrow cultures (LTBMC) have proved useful for modelling the structure of the stromal microenvironment, and the various interactions between the stromal cells and the haemopoietic stem cells. Indeed, within the bone marrow, the stem cells reside in regulatory niches consisting of various cell types such as endothelial cells, fibroblasts, adipocytes and macrophages. Stromal cells not only produce growth factors, they also produce a wide range of extracellular matrix molecules (ECM) such as, collagens, fibronectin, tenascin and cellular adhesion molecules (CAMs) e.g. I-CAM-1, VCAM-1, and integrins (VLA-4) which provide a supportive milieu for the stem cells allowing them to function.

The extracellular components of the multiple cell types such as fibronectin, collagen and tenascin-C are reported to play a role in the interaction of the HSC and the stroma. Indeed, antibodies specific for fibronectin have been observed to inhibit the formation of CFU-S d12 in LTBMC (Williams *et al* 1991c), and tenascin-C specific antibodies have the ability to block the adhesion of HPCs to stroma (Klien *et al* 1993). Fibronectin has also been observed to increase the production of CFU-E, BFU-E and CFU-GEMM colonies derived from human bone marrow, and can further increase their formation in the presence of IL-3 (Zhou *et al* 1993). Further evidence for the role of these ECM proteins was observed upon the analysis of their respective null mice. Fibronectin null mice are unable to form blood islands in the embryo and are therefore embryonic lethal (George *et al* 1993) whereas, Tenascin-C null mice are viable and have a reduced level of haemopoiesis, suggesting that Tenascin C may play a role in the interactions between the haemopoietic stem cells and the stromal cells

(Ohta *et al* 1998). These studies indicate that the interaction of stem cells and stromal cells via ECM molecules may be important in the regulation of stem cell functions, and are reviewed by Whetton and Graham (Whetton and Graham 1999).

Glycosaminoglycans such as heparin, heparan sulphate and chondroitin sulphate are expressed on stromal cells and the various populations of stem/ progenitor cells, and are thought to be involved in interactions between these cells. Indeed, heparan sulphate (HS) has been observed to be involved in the adhesion of primitive haemopoietic progenitor cells (Siczkowski *et al* 1992), and has been observed to bind both growth stimulating and inhibiting factors, such as IL-3, GM-CSF and TGF-β, (Roberts *et al* 1988a, Lopez-Casillas *et al* 1993). Furthermore, a report by Gupta *et al* indicated that the HS in combination with cytokines could maintain levels of human LTCIC cells in long-term bone marrow cultures (Gupta *et al* 1996). Therefore, these reports suggest that HS may regulate stem cell activity by being involved in co-localising progenitors with heparin binding cytokines in the ECM or alternatively they may act as a presentation molecule for various growth factors such as fibroblast growth factor (Ornitz *et al* 1992, Faham *et al* 1998).

A further type of molecule involved in interactions between the stem cells and the stromal cells within the bone marrow microenvironment are adhesion molecules. Both stromal cells and HSCs have been observed to express large number of CAMs, including members of the integrin superfamily (VLA-4, 5), the sialomucin family (CD34, CD45RA, CD43 and CD164) and immunoglobulin family (CD31 and CD50), as well as ligands for selectins (Simmons *et al* 1997). Evidence for the role of these adhesion molecules in regulating haemopoiesis was initially observed by the fact that antibodies to VLA-4 added to LTBMC abrogated lymphopoiesis, reduced CFU-S d12 cell production and the level of myelopoiesis (Williams *et al* 1991c, Miyake *et al* 1991). Antibodies to VLA-4 have also been observed to mobilise stem cells to the peripheral blood (Craddock *et al* 1997), and B cell precursors have been shown to adhere to bone marrow fibroblast through the interaction of VLA-4 and VCAM-1 (Ryan *et al* 1991). Furthermore, the disruption of the α4-integrin gene was observed to lead to impaired T and B lymphopoiesis in mice (Arroyo *et al* 1996). These results suggest that VLA-4 plays a major role in the interaction between HSC and stromal cells. Other studies have also indicated a role of adhesion molecules in the bone marrow microenvironment. Indeed, Bazil and colleagues suggested that mucin like

molecules may function as negative regulators of haemopoiesis. They observed that antibody mediated cross linking of the mucin CD43 induced apoptosis in the $CD34^+$ haemopoietic progenitor cells, and that the multipotential (CFU-GEMM) and erythroid progenitors (BFU-E) were more sensitive than committed progenitors (CFU-M) (Bazil *et al* 1995). Furthermore, another member of the mucin family CD164 is also involved in adhesion of HSC to stroma (Watt *et al* 1998) and was found to induce apoptosis and suppress the recruitment of quiescent haemopoietic stem cells into cell cycle (Zannettino *et al* 1998). Thus, these various studies indicate the important role of adhesion molecules in the regulation of stem cell function within the bone marrow microenvironment.

In addition to providing an adherent surface for the stem cell to sit down on, the stem cell niche produces a range of soluble and transmembrane localised growth factors and their receptors, such as SCF, M-CSF, *c-kit* and *c-fms*. The interaction of these growth factors and their receptors is not only involved in stem cell function, they also display a range of activities on a number of additional cell types and these are discussed in the next section.

1.7 Growth Factors

The continual production of blood cells during an individuals lifetime, is mediated by the co-ordinated effects of a number of cytokines and growth factors acting on the haemopoietic stem and progenitor cells within the bone marrow. The small number of haemopoietic stem cells can proliferate and differentiate into lineage committed progenitors which eventually generate all the different kinds of mature terminally differentiated blood cells. Among such growth factors, SCF and FL are involved in survival of early haemopoietic stem cells, interleukin-3 and GM-CSF stimulate multi-lineages of haemopoietic cells while M-CSF, G-CSF and EPO stimulate more restricted lineages of the haemopoietic system. Each of these growth factors and their actions within haemopoiesis are described in the sections below, as SCF, M-CSF and GM-CSF are involved in CFU-A colony growth these have been described in more detail.

1.7.1 Tyrosine Kinase Signalling Growth Factors

1.7.1.1 Stem Cell Factor (SCF)

Stem Cell Factor (SCF) is the gene product of the murine Steel (*Sl*) locus and the ligand for the *c-kit* tyrosine kinase receptor, the product of the dominant white spotting locus (*W*). Despite the *Sl* locus being localised to chromosome 10 and the *W* locus on chromosome 5 defects at these genetic loci result in similar phenotypes. These are characterised by white spots on the bellies of pigmented mice, which is due to the inability of the melanocytes to migrate to the hair follicle. Furthermore, these mice have reproductive difficulties and are anaemic, due to the ineffective migration of the germ cells and the reduction in the number of erythrocytes respectively. Due to similar phenotypes produced by these separate mutations researchers hypothesised that there was a relationship between these two loci. Indeed, in 1988 two groups found that the *W* locus encoded a tyrosine kinase receptor known as *c-kit* (Chabot *et al* 1988, Geissler *et al* 1988), and subsequently the cDNA corresponding to SCF was isolated from different sources, this lead to the protein being named in accordance to its source, mast cell growth factor (MGF), stem cell factor (SCF) and *c-kit* ligand (Huang *et al* 1990, Martin *et al* 1990, Williams *et al* 1990b). Within this thesis this protein will be referred to as SCF. Although the mouse and human proteins are 82 % identical at the amino acid level they show varying degrees of species specificity indeed, the full-length mouse protein is active on human cells whereas, the human SCF has limited activity on murine cells (Martin *et al* 1990). As a results of alternative splicing there are two major isoforms of SCF in mice and humans, a membrane associated glycoprotein of 248 amino acids (aa) which is rapidly cleaved to release a biologically active soluble protein of 164 aa and a glycoprotein of 220 aa which lacks the proteolytic cleavage site encoded in exon 6, and thus remains predominantly membrane associated. Interestingly, this isoform can also be slowly released from the cell surface through the use of an alternative proteolytic cleavage site (Flanagan and Leber 1990). More recent studies have indicated that proteolytic enzymes from mast cells can cleave SCF thus producing alternative SCF products of various lengths. This was hypothesised to be a possible method of regulating the activity of SCF on the mast cells or may even allow the production of another SCF

isoform that has distinct activities on these mast cells (Longley *et al* 1997, dePaulis *et al* 1999).

Stem cell factor can be expressed by various different cell types such as fibroblasts, stromal cells, keratinocytes, gut endothelial cells and the cells of the central nervous system (Longley *et al* 1993, Linenberger *et al* 1995, Klimpel *et al* 1995, Zhang *et al* 1998a). Interestingly, Huang *et al* observed that there is tissue specific expression of the different SCF isoforms and thus hypothesised that SCF function may differ depending upon whether the target cell expressing the SCF receptor interacts with the soluble or membrane bound isoforms (Huang *et al* 1992). The analysis of the phenotypic abnormalities of *Steel-Dickie* (Sl^d) mice together with analysis of the biological activity of the soluble and membrane forms of SCF have indicated that the membrane form of SCF is necessary for normal phenotype in these mice (Toksoz *et al* 1992). Furthermore Toksoz *et al* also observed that membrane associated SCF supported long term production of primitive haemopoietic progenitors *in-vitro*, whereas only a transient maintenance of haemopoiesis was observed with soluble SCF (Toksoz *et al* 1992). Further evidence for the distinct roles of membrane bound and soluble forms of SCF was reported in transgenic Sl/Sl^d mutant mice expressing either soluble or membrane restricted SCF. Indeed, the membrane restricted SCF isoform could partially correct the anaemia associated with these mice whereas the soluble form could not, however, in contrast the soluble SCF isoform and not the membrane restricted form was observed to restore the myeloid progenitor cell numbers (Kapur *et al* 1998, 1999).

SCF is a multifunctional cytokine that displays activities on mast cells, melanocytes germ cells, and a whole number of haemopoietic cells such as stem cells, progenitor cells and mature cells. SCF has the ability to act as a survival factor in the absence of other growth factors. Indeed, SCF inhibits apoptosis in NK cells, murine myeloid and mast cell lines (Carson *et al* 1994, Gommerman *et al* 1998) and also allows the survival of both murine and human primitive haemopoietic stem cells (Keller *et al* 1995, Borge *et al* 1997). SCF is widely expressed in embryogenesis and along with its receptor *c-kit* they play a role in the migration and the correct tissue localisation of primordial germ cells, melanocytes and hematopoietic stem cells (Matsui et al 1990, Keshet et al 1991). Although SCF acts alone in the survival of stem and progenitor cells there are numerous studies that indicate that SCF acts in combination with

various growth factors in a wide range of functions. Indeed, SCF can act together with IL-3 to expand the numbers of progenitor cells, such as BFU-E, CFU-GM and colony forming unit granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) by 200 fold in liquid culture (Brugger *et al* 1993). Furthermore, culturing of murine marrow cells in a cytokine cocktail (SCF, IL-3, IL-6 and IL-11) expanded the number of progenitor cells, low proliferative potential colony forming cells (LPP-CFC) and HPPCFC, but impaired the long term repopulating ability (Peters *et al* 1996). As well as SCF synergy with various growth factors in the expansion of cell numbers, SCF also synergises with EPO, IL-3, GM-CSF, TPO, IL-6, IL-7 and G-CSF to increase both size and number of BFU-E, CFU-GM, CFU-GEMM and CFU-Meg colonies in semisolid media. However, alone SCF has only modest effects on colony growth (Avarham *et al* 1992, Briddell *et al* 1991, Tanaka *et al* 1992, Fahlman *et al* 1994).

A further important function of SCF is its ability to induce adhesion and migration and, these functions are important for determining the correct tissue localisation of cells. Indeed, SCF is a potent stimulator of the adhesion of mast cells, haemopoietic progenitor cell lines and $CD34^+$ progenitors to fibronectin and vascular cell adhesion molecule-1 VCAM-1 (Levesque *et al* 1995, Dastych *et al* 1994, Kodama *et al* 1994). Membrane bound SCF may also act as an adhesion molecule for mast cells and haemopoietic cells and may explain why the incubation of these cells with antibodies to *c-kit* reduces their homing efficiency (Broudy *et al* 1996). Furthermore, SCF has been observed to mobilise human and murine haemopoietic stem cells from the bone marrow to the peripheral blood and also displays chemotactic and chemokinetic properties on various HPC in $Sca\text{-}1^+$ / Lin^- cells (Okumura *et al* 1996).

SCF activities are not restricted to haemopoietic cells indeed, membrane bound SCF has been observed to be involved in the maintenance and proliferation of liver stem cells and the expression of soluble SCF and its receptor have been observed during liver regeneration in rats (Fuijo *et al* 1994). Furthermore, Zhang *et al* also observed that both soluble and membrane associated SCF can be expressed by neurones and it appears that the membrane form is more important for microglial cell survival in neuron-microglia mix cultures (Zhang and Fedoroff 1997).

As is the case with SCF, *c-kit* the SCF receptor, also exists as different isoforms one of which is lengthened by four amino acids (glycine-asparagine-asparagine-lysine). Both receptors are ligand-induced protein tyrosine kinase (PTK), which belong to the receptor subfamily III, members of which include the receptors for EGF, PDGF, *c-fms* and *flt3*/FLK2 (Hanks *et al* 1988). As well as these isoforms, a soluble *c-kit* receptor has been found at high levels in human serum and in the culture medium of some haemopoietic cell lines (Turner *et al* 1995, Wypych *et al* 1995). The observation that *c-kit*, like SCF, has differing isoforms indicates that the various interactions between these isoforms may be a further method for regulating the various functions of SCF at the cellular level. Interestingly, the smaller of the *c-kit* isoforms has been reported to be constitutively active producing a low level of auto-phosphorylation. This basal level of activation may be enough to promote survival of some cell types and higher levels produced when SCF is present may produce an alternative signal (Reith *et al* 1991).

SCF receptor, *c-kit*, can be detected on a host of cell types such as mast cells, promyelocytes, myelocytes, eosinophils, monocytes, microglial and primordial germ cells (Metcalf 1991a, Fukada *et al* 1995). Within the haemopoietic stem cell compartment *c-kit* was initially observed to be expressed by all the *in-vitro* clonogenic cells with the exception of pre-B progenitor colony forming cells responsive to IL-7 (Ogawa *et al* 1991). However, more recent studies on human haemopoietic progenitor cells have indicated that there is a population of cells that display a *c-kit*$^-$ phenotype. This *c-kit*$^-$ cellular population were observed to possesses long term repopulating activity and upon growth factor stimulation became *c-kit*$^+$ cells, thus suggesting that the expression of *c-kit* on *c-kit*$^-$ cells may be the first maturational step of haemopoiesis for these primitive human progenitor cells (Sogo *et al* 1997). Two further studies in the mouse also isolated a *ckit*$^-$ stem cell population that displays long term repopulating activity (Doi *et al* 1997, Ortiz *et al* 1999). Thus collectively, these studies suggest that although human and murine LTRC were initially observed to express high levels of cell surface *c-kit*, it seems that they coexist with a less frequent sub population of LTRC with undetectable *c-kit* expression and it is therefore, possible that the expression of *c-kit* on these LTRC cells is the first step in maturation of this population of long term repopulating cells. Therefore, the expression of *c-kit* receptor whether it is an increase in PHSC or a decrease in

progenitor cells indicates that stem cell factor and its receptor play an important role in the maturation of cells at various stages of the haemopoiesis.

1.7.1.2 Macrophage Colony Stimulating Factor (M-CSF)

Macrophage colony stimulating factor (M-CSF), or colony stimulating factor-1 (CSF-1) as it was originally known, was initially identified as a haemopoietic growth factor that stimulates haemopoietic precursors to form colonies containing mononuclear phagocytes *in-vitro* (Stanley and Heard 1977). Unlike the granulocyte/macrophage-colony stimulating factor (GM-CSF) and Interleukin-3 (IL-3), which also directly induce mononuclear phagocyte proliferation, M-CSF is a lineage specific growth factor which plays an essential role in the survival and maturation of the mononuclear phagocytic lineage (Stanley *et al* 1983).

Biologically active, secreted and membrane bound forms of M-CSF (Rettenmeir *et al* 1988, Wong *et al* 1987, Manos *et al* 1988) are encoded by alternatively spliced messenger RNA's translated from a single unique gene which is located on chromosome 3 in mouse and chromosome 5 in humans (Kawasaki *et al* 1985, Morris *et al* 1991). Combining differential splicing and complex co and post-translational modifications the M-CSF mRNA species generates different mature M-CSF isoforms: a homodimeric secreted M-CSF glycoprotein of 85 kDa (Kawasaki *et al* 1985) which is the main form of M-CSF detected in body fluids (Suzu *et al* 1994), a membrane associated homodimeric 68 kDa form (Uemura *et al* 1993) which can also be slowly released as a soluble molecule of 44 kDa, and a homodimeric or heterodimeric M-CSF proteoglycan (PG-M-CSF) of anything up to 150 kDa (Suzu *et al* 1997). Different cell types under various stimuli have been observed to produce different ratios of soluble and membrane associated M-CSF and thus may suggest differences in the functions of these M-CSF isoforms.

M-CSF has been observed to be detected in blood and bone marrow plasma (Suzu *et al* 1994) and *in-vitro* M-CSF can be produced by a variety of cell types such as endothelial cells, thymic epithelial cells, monocytes-macrophages, marrow stromal cells, B and T cells, osteoblasts, astrocytes, microglia, neurones and keratinocytes (Seelentag *et al* 1987, Horiguchi *et al* 1986, Oster *et al* 1987, Fixe *et al* 1997, Praloran 1991, Alterman *et al* 1994, Hallet *et al* 1991). Furthermore, these cells can be

induced to express M-CSF by various factors such as interferon gamma (IFN-γ), tumour necrosis factor (TNF α), IL-1, and GM-CSF (Praloran *et al* 1991, Hashimoto *et al* 1997).

As stated earlier, M-CSFs main activities are observed on the various cells of the mononuclear phagocytic lineage, indeed M-CSF can enhance the differentiation of monocytes to macrophages and this can be observed by the induced expression of surface antigens such as HLA-DR, Fc-receptor and CD11b (Hashimoto *et al* 1997). M-CSF potentiates the ability of macrophages to kill infectious microorganisms (Karbassi *et al* 1987, Lee *et al* 1987) and tumour cells (Wing *et al* 1982). Furthermore, M-CSF enhances these functions by the induction of macrophage cytokines such as interferon, TNF, IL-1, GM-CSF (Moore *et al* 1980, 1984, Warren and Ralph 1986, Motoyoshi *et al* 1982) as well as other inflammatory mediators, such as prostaglandins and chemokines (Kurland *et al* 1978, Hashimoto *et al* 1996, Lyberg *et al* 1987). Thus M-CSF not only activates macrophage directly it also primes these cells so that they can respond to other stimuli. A further interesting function of M-CSF was observed by Sakurai *et al*, they demonstrated that a single injection of M-CSF into mice produced an increase in the number of natural killer (NK) cells in the spleen, and subsequent injections of M-CSF increased the NK activity of these mononuclear cells (Sakurai *et al* 1997). Further evidence to suggest that M-CSF is involved in regulating the cytoxicity of mononuclear cells was obtained when Jadus *et al* observed that the expression of M-CSF on the cell surface of tumour cells inducing macrophages to recognise and kill these tumour cells bearing the M-CSF (Jadus *et al* 1996). This suggested that M-CSF may have a role in cancer treatment however, contradictory reports indicated that M-CSF has no benefits in the treatment of cancer (Dorsch *et al* 1993)

M-CSF activities are not only restricted to mature cell populations in the mononuclear phagocytic lineage. M-CSF can stimulate the formation of murine macrophage monocytic progenitor colonies (CFU-M), and in combination with other growth factors it can also stimulate the formation of colonies from primitive cells such as high proliferative potential colony forming cells (HPP-CFCs) (Bartlemaz 1989) and colony forming unit-agar (CFU-A) cells (Pragnell *et al* 1988, Holyoake *et al* 1993). Further evidence for the role of M-CSF in haemopoietic stem and progenitor colony growth was observed by the inhibition of the formation of CFU-A and day 12 colony

forming unit spleen CFU-S d-12 colonies by neutralising antibodies to the M-CSF receptor, *c-fms* (Gilmore *et al* 1995). Furthermore, Muench *et al* observed that M-CSF can stimulate colony growth of primitive fetal liver colony-forming stem cells, $CD34^+$ / $CD38^+$/ Lin^- (Muench *et al* 1997).

M-CSF has activities outwith the haemopoietic system indeed, Kawada *et al* reported that M-CSF as well as G-CSF stimulated the proliferation of human keratinocytes (Kawada *et al* 1997). Michaelson *et al* also provided data to suggest that M-CSF is an important factor in CNS development (Michaelson *et al* 1996). Further biological roles of M-CSF have been observed in the mutant osteopetrotic (*op/op*) mice, a natural occurring M-CSF mouse knockout. These *op/op* mice have a reduced level of macrophages and osteoclasts, they are infertile and are osteopetrotic due to a reduced level of bone remodelling (Wiktor-Jedrzejczak *et al* 1990, 1991, Pollard *et al* 1987,1991). These conditions are a direct consequence of the loss of M-CSF, as the addition of recombinant M-CSF can correct these abnormalities which are also progressively corrected as the mice age (Begg and Bertoncello 1993). The characteristic defects of the *op/op* mice can also be reversed by the addition of GM-CSF or IL-3 (Myint *et al* 1999). This correction of macrophage deficiency on the treatment of *op/op* mice with GM-CSF and IL-3 indicates that there is an overlap in the functions with these growth factors. On further analysis of these *op/op*, Michaelson *et al* observed that the loss of M-CSF lead to abnormal brain development in these mice thus, suggesting a role of M-CSF in the central nervous system (Michaelson *et al* 1996).

The various actions that M-CSF exerts on the numerous target cell types is mediated through its interaction with its receptor, *c-fms* which is alternatively known as CD 115 (Sherr *et al* 1985). Like *c-kit, c-fms* is a member of the receptor subfamily III that exhibit ligand-induced tyrosine kinase activity (Hanks *et al* 1988). The *c-fms* proto-oncogene maps near the M-CSF locus on the human chromosome 5 at band 5q33.3 (Roussel *et al* 1983, Groffen *et al* 1983), and is linked in tandem with the type β PDGF-receptor gene (Roberts *et al* 1988b). In this location there are several other growth factor genes that play important roles in haemopoiesis, these include GM-CSF, IL-4 and IL-5 genes (Le Beau 1986, Sutherland *et al* 1988a, b). Both human and mouse M-CSF receptors are integral transmembrane glycoproteins and share 74% overall homology, the greatest sequence homology occurring in the intracellular

kinase domains. Within the haemopoietic system *c-fms* has been observed to be expressed on macrophages, blast cells, myeloid precursors, lymphocytes and on cells of the megakaryocyte or erythroid lineage (Byrne *et al* 1981). Furthermore, the level of *c-fms* expression is thought to be dependant on the maturation state of the cell as suggested by the observation that *c-fms* mRNA is induced in the HL60 cell line after the induction of differentiation by phorbol myristate acetate (Biskobing *et al* 1993, Stone *et al* 1990, Perkins and Kling 1995). The M-CSF receptor expression is not restricted to the cells of the haemopoietic system as it has been observed to be expressed on placental trophoblasts (Regenstrief and Rossant 1989), osteoclasts (Hofsetter *et al* 1992) and on macrophage-like microglial cells in the nervous system (Sawada *et al* 1990, Brosnan *et al* 1993). Indeed, Raivich *et al* demonstrated that the macrophage like cells of the CNS, the microglial cells, express low levels of *c-fms*. The level of *c-fms* expression is induced upon CNS injury therefore, suggesting that the level of *c-fms* expression may have a role in preparing the microglial to take part in the cellular response involved in CNS injury (Raivich *et al* 1998).

1.7.1.3 Flt Ligand (FL)

Flt ligand (FL) appears to play a role in the functions of the haemopoietic stem and progenitor cells, and similar to SCF it also displays various synergistic activities with other growth factor on a range of haemopoietic cells.

Due to alternative splicing FL exists as a membrane bound and a soluble form, both of which exhibit similar biological activities (Lyman *et al* 1995a). FL is not species specific and this is thought to be due to the high degree of homology shared between the human and murine FL proteins, 72 % identity at the amino acid level (Lyman *et al* 1993, Broxmeyer *et al* 1995). In normal individuals the plasma levels of FL are low and can only be detected by sensitive ELISA however, these levels have been observed to be increased in haemopoietic disorders such as acquired aplastic anaemia (Lyman *et al* 1995b) and thus is thought to play a role in this disorder.

In-vitro studies have indicated that, like SCF, FL can support maintenance of stem and progenitor cells (Hudak *et al* 1995, Muench *et al* 1995). Initially it was observed that FL alone had no significant colony stimulating activity however, subsequent studies indicated that FL alone or in combination with GM-CSF, M-CSF, IL-3, IL-6,

IL-11, IL-12 and SCF enhanced the proliferative response or colony forming potential of purified human and murine stem and progenitor cells (Muench *et al* 1995, Jacobsen *et al* 1995a, Rasko *et al* 1995). Further reports have indicated that FL functions are not restricted to the myeloid lineage as FL in combination with IL-7 acts on the lymphoid lineage (Muench *et al* 1995). FL has also been observed to be an effective replacement for SCF in *ex-vivo* expansion protocols as it has been observed to expand $CD34^+$ progenitors (Mckenna *et al* 1995) namely CFU-GM and CFU-GEMM progenitors (Broxmeyer *et al* 1995).

Flt-3 ligand (FL) induces its haemopoietic growth factor activities through interaction with its specific receptor, *fms*-like tyrosine kinase 3 *(flt3)* also referred to as fetal liver kinase 2 (FLK-2). While expression studies have indicated that FLK2/ *flt3* ligand (FL) is ubiquitously expressed (Lyman *et al* 1995a) it seems that its receptor is more restricted to primitive haemopoietic progenitors (Small *et al* 1994). FLK-2/*flt3* is a member of the tyrosine kinase receptor family, which includes M-CSF receptor and SCF receptor and is, located on mouse chromosome 5 and human chromosome 13 (Rosnet *et al* 1993).

Targeted disruption of the *flt3*/ FLK2 or FL gene produces normal healthy mice that have reduced levels of B cell progenitors (Mackarehtschian *et al* 1995, McKenna *et al* 1996, 2000) and further studies have also reported that FL deficient mice have reduced levels of dendritic and NK cells (McKenna *et al* 2000). These studies indicate that although FL is involved in various lineages, other growth factors can compensate for the loss of FL activity. Interestingly mice that lack both *flt3*/FLK2 and *c-kit* die after birth, and on analysis of their haemopoietic systems it was observed that these animals had a severe reduction in their overall size of the haemopoietic system, particularly in the myeloid and lymphoid lineages and a reduction in all progenitor cells. These results indicate that FL may play a role in haemopoietic development (Mackarehtschian *et al* 1995) and that FL is part of a network of cytokines that regulate the growth and survival of early haemopoietic stem and progenitor cells.

1.7.2 Beta Common (βc) Signalling Growth Factors

1.7.2.1 Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

Granulocyte macrophage colony stimulating factor (GM-CSF) is a haemopoietic growth factor that derives its name from the ability to stimulate the formation of *in-vitro* macroscopic colonies containing neutrophils, eosinophils, and macrophages, or a mixture of these cell types. Historically growth factors such as GM-CSF were purified from conditioned medium that displayed the ability to stimulate the growth of colonies from immature bone marrow derived progenitors (Broxmeyer *et al* 1990, 1999). Indeed, murine GM-CSF was no exception as it was first identified and purified from mouse lung conditioned medium (Burgess and Metcalf 1977). Around the same time Weisbart *et al* identified a factor that could inhibit the migration of neutrophils and named it NIF-T (Weisbart *et al* 1979). Human GM-CSF was subsequently purified from a human T-cell leukaemia virus type II (HTLV-II)-infected T-lymphoblastoid cell line, Mo. However, it wasn't until 1985 that Wong *et al* cloned human GM-CSF and NIF-T and GM-CSF were observed to be identical and are now known as GM-CSF (Wong *et al* 1985).

The GM-CSF gene has been mapped to the long arm of chromosome five between 5q21-32, in an area containing several other haemopoietic growth factors and their receptors. The full length human GM-CSF is 127 amino acids in length and has a molecular mass of approximately 22 kDa (DiPersio *et al* 1988) and although human and murine GM-CSF share 60 % identity they are not cross reactive.

There are a number of different cell types that produce GM-CSF following various kinds of stimuli. Indeed, GM-CSF production has been demonstrated in monocyte/macrophages, neutrophils, B-lymphocytes, eosinophils, mast cells, keratinocytes, osteoclasts as well as different epithelial cells. Further expression studies have reported that the constituent cells of the bone marrow microenvironment produce GM-CSF (Kita *et al* 1991, Baldwin *et al* 1992, Jung *et al* 1995). However, most of the GM-CSF expressing cells under resting conditions are not significant sources of GM-CSF, but rapidly and transiently can be stimulated by factors such as phorbol esters, interferon-γ, LPS, IL-1 and TNF-α to produce GM-CSF (Kothori *et al* 1995). Therefore, it seems that the activation state of the cells is the major factor in

determining whether the cells that have the potential to express GM-CSF actually do. In contrast to other members of the CSF family such as G-CSF, GM-CSF cannot be detected in the circulation by ELISA, and this may be due to GM-CSF being sequestered by the extracellular matrix (Modrowski *et al* 1998).

GM-CSF was the first human colony stimulating factor to be purified, cloned and expressed using recombinant DNA technology. This allowed vast amounts of GM-CSF to be synthesised which lead to the further investigation into the functions of GM-CSF. There is evidence to suggest that GM-CSF plays a role in immune responses, GM-CSF can stimulate the cytotoxic capacity of neutrophils and monocytes (Masucci *et al* 1989), expand and activate antigen presenting cells (APCs), induce the differentiation of monocytes to macrophages (Inaba *et al* 1992, Sallusto and Lanzavecchia 1994), activate immune functions in eosinophils and basophils (Fabian *et al* 1992, Negata *et al* 1995) and increase natural killer cell function. Furthermore, GM-CSF can induce the expression of growth factors and cytokines such as M-CSF, G-CSF, IL-12 and IL-15 and other pro-inflammatory agents such as prostaglandin's, plasminogen activators, interferon-γ, TNF and IL-8 from the above cells (Takahashi *et al* 1993, Bendall *et al* 1995). GM-CSF can therefore directly or indirectly affect the functions of various mature cells (Yong and Linch 1993, Weiser *et al* 1987, van Pelt *et al* 1996). GM-CSF not only stimulates the growth of various mature cell types such as granulocytes, dendritic cells, Langerhans cells, eosinophils, megakaryocytes, it can also stimulate the proliferation of progenitors such as CFU-G, CFU-M, CFU-E and CFU-GM from human and murine bone marrow or human peripheral blood (Broxmeyer *et al* 1990, 1999, DeWynter *et al* 1998).

The high affinity receptor for GM-CSF is composed of two sub-units, the α and β sub-unit (Hayashida *et al* 1990, Tavernier *et al* 1991, Kitamura *et al* 1991). The α sub-unit is specific for GM-CSF and binds it with low affinity whereas the beta (βc) sub-unit is shared between GM-CSF, IL-3 and IL-5 (Matsuguchi *et al* 1997). To date, there are at least six isoforms described for the GM-CSFR alpha (Chopra *et al* 1996). Humans have only one β sub-unit (βc), which has no binding capacity by itself but can form a high affinity IL-3, IL-5 and GM-CSF receptors with their respective α sub-units. Both sub-units belong to the cytokine receptor family, which includes the receptors for many haemopoietic growth factors and cytokines such as growth

hormone, erythropoietin (EPO), thrombopoietin (TPO), IL-2 and IL-6 (reviewed by Mui 1994). Members of this family are characterised by an extracellular cytokine receptor module (CRM) of about 200 amino acids containing several conserved motifs, including the hallmark WSXWS (Trp-Ser-Xaa-Trp-Ser) motif and a cytoplasmic domain that lacks any intrinsic enzymatic activity associated with signal transduction (Bagley *et al* 1997). Although only the βc sub-unit has the potential to produce a signal, truncation studies have indicated that the interaction between the α and the βc sub-unit is neeeded for the activation of JAK/STAT signalling pathways (Scott *et al* 1998, Doyle *et al* 1998). Neither the α or β sub-unit have intrinsic kinase domains or are linked to G-proteins, instead the receptor produces a signal by associating with and activating a number of cytosolic tyrosine kinases including *lyn*, *fes* and JAK2 (Thompson *et al* 1995, Linnekin *et al* 1995, Al-Shami *et al* 1997, 1998). GM-CSFRα can exist in both a transmembrane form and a soluble form indeed, naturally occurring soluble forms of sGM-CSFRα can be detected in the supernatants of human neutrophils and in human plasma and may allow GM-CSF to interact with cells that do not express the membrane form of the GM-CSFRα sub-unit (Sayani *et al* 2000).

There is some debate about the expression of the GM-CSF receptor in the primitive cells of the haemopoietic system. Several studies have suggested that primitive progenitors lack the receptor for GM-CSF (Wognum *et al* 1994, Jubinsky *et al* 1994, Berardi *et al* 1995). Indeed, McKinstry *et al* observed that GM-CSF receptor could not be found on primitive populations but could be detected on committed progenitors cells that were defined as Sca-1^+/c-kit^+ (McKinstry *et al* 1996). In contrast, a study by Lund-Johansen *et al* observed that a primitive human haemopoietic progenitor population, which is defined by the expression of CD34hi CD38lo, expresses both the alpha and beta sub-units of the GM-CSF receptor and that GM-CSF displayed minimal effects on the survival or proliferation of these primitive progenitors. However, in combination with the SCF, GM-CSF can enhance the survival and growth of these cells (Lund-Johansen *et al* 1999). These differences may be due to the isolation of different primitive cell types in these reports.

The *in-vivo* effects of GM-CSF have been examined in a number of ways in murine models; by injecting GM-CSF (Metcalf *et al* 1987), by generating hGM-CSF receptor

transgenic mice (Nishijima *et al* 1997), by reconstituting mice with bone marrow cells that over produce GM-CSF (Lang *et al* 1987) or examining GM-CSF or GM-CSFR null mice. Indeed, Stanley *et al* produced GM-CSF null mice and on examining these animals they observed that there was no perturbation of the major haemopoietic populations in blood or bone marrow (Stanley *et al* 1994). Thus indicating that other regulators can replace GM-CSFs activity in its absence however, GM-CSF is not totally redundant as indicated by the formation of the abnormal lung pathology, pulmonary alveolar proteinosis (PAP). This lung pathology is due to the malfunctioning of alveolar macrophages and can be cured by a bone marrow transplant (Nishinakamura *et al* 1996). A similar pulmonary phenotype was observed on the examination of mice lacking the βc receptor sub-unit of the GM-CSF receptor, which is also common to the IL-3 and IL-5 receptors. These mice also displayed a reduction in eosinophils, which was consistent with the loss of IL-5 activity, one of the major growth factors necessary for eosinophil development and response (Nishinakamura *et al* 1995, Stanley *et al* 1994). This pulmonary alveolar proteinosis (PAP) condition has also been observed in humans, however, on analysis of their bone marrow cells it was observed that they have both the GM-CSFRα and βc sub-units of the GM-CSF receptor. Furthermore, GM-CSF administration could not reduce the disease, suggesting that the GM-CSF signalling system is not wholly responsible for the pathology of this disease (Carraway *et al* 2000)

1.7.2.2 Interleukin-3 (IL-3)

IL-3 is a multipotential haemopoietic growth factor capable of stimulating the survival, proliferation and development of multipotent stem cells and myeloid progenitor cells. In contrast to many other growth factors, IL-3 expression is restricted to only a few cell types. Furthermore, Gibson *et al* indicated that IL-3 appeared not to be produced in normal animals however, IL-3 expression can be observed but only with the use of sensitive technique such as RT-PCR (Gibson *et al* 1995). In peripheral blood the predominant IL-3 expressing cells are antigen or mitogen stimulated T cells. Other reports have also observed that NK cells, megakaryocytic cells, epithelial and mast cell lines can express IL-3 (Gibson *et al* 1995, Cuturi *et al* 1989, Wickenhauser *et al* 1995, Nimer *et al* 1995, Wodnar-Filipowicz *et al* 1989, Dalloul *et al* 1991). The restricted production of IL-3 and the inability to detect levels of it in serum and bone marrow with out the aid of sensitive techniques has lead to the

proposal that IL-3 may not be involved in the day to day control of haemopoiesis (constitutive haemopoiesis), and that it may only be needed in times of stress or infection (inducible haemopoiesis). This is supported by the need for activation of T cells, mast and megakaryocytic cell lines before they express IL-3 (Gibson *et al* 1995, Nimer *et al* 1995).

In-vitro IL-3 has been observed to support the proliferation of $CD34^+$ bone marrow and myeloid progenitors cells and also supports the development of these cells down the megakaryocytic and erythroid lineages however, terminal differentiation only occurs in the presence of TPO or EPO (Banu *et al* 1995, Saeland *et al* 1988). Furthermore, IL-3 has been observed to be involved in the differentiation of eosinophils (Saito *et al* 1988) and can interact with other growth factors such as SCF to promote mast cell proliferation (Rennick *et al* 1995). IL-3 activities are not only restricted to supporting growth and differentiation as in conjunction with SCF, M-CSF or the CXC chemokine SDF-1, IL-3 has been observed to display chemotactic and chemokinetic responses on murine haemopoietic progenitors (Okumura *et al* 1996, Aiuti *et al* 1997). Other reports have also suggested that IL-3 may also be involved in regulating adhesion of stem cells to the stromal cells. Indeed, IL-3 can reduce integrin mediated adhesion and promote migration of $CD34^+$ cells (Schofield *et al* 1997) and this may explain the enhanced mobilisation of peripheral blood progenitors by IL-3 in the presence of G-CSF (Huhn *et al* 1996). A further activity of IL-3 was observed using a multipotential progenitor cell line, FDCP-Mix, these cells depend on IL-3 for growth and upon its removal these cells undergo apoptosis, indicating that IL-3 acts as a survival factor for this cell line (Williams *et al* 1990a, Fairbairn *et al* 1993). These reports indicate that *in-vitro* IL-3 can regulate the functions of various cell types at different stages of maturation.

As alluded to in section 1.7.2.1, IL-3 induces its activities through a common signalling sub-unit shared by IL-5 and GM-CSF, the βc sub-unit (Mui *et al* 1994). In mice IL-3 can also signal through an IL-3 specific beta sub-unit namely $\beta^{IL\text{-}3}$ (Hara *et al* 1992, 1996). The βc and $\beta^{IL\text{-}3}$ sub-units are expressed on myeloid progenitor cells, macrophages, mast cells, B cells and endothelial cells whereas, the expression of the alpha sub-unit is more restricted suggesting that IL-3 responses are regulated by the level of IL-3α sub-unit expression. Mice that completely lack IL-3 have been

observed to display no aberrant phenotype suggesting that other growth factor can compensate for the loss of IL-3 function (Nishinakumura *et al* 1996). Furthermore, the observation that there are certain strains of laboratory mice that have no IL-3Rα expression and have no alteration in their haemopoietic system, indicates that the IL-3 system is not essential for normal haemopoiesis and the role IL-3 plays in haemopoiesis *in-vivo* has yet to be defined (Nicola *et al* 1996).

1.7.3 Glycoprotein 130 (gp130) Signalling Growth Factors

1.7.3.1 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) was originally identified as a factor that induces B cell terminal differentiation into antibody producing cells (Hirano *et al* 1986). It was subsequently observed to display numerous activities, which include growth promotion, haemopoietic colony formation, differentiation of macrophages and T cells, neural differentiation and induction of acute phase proteins (Simpson *et al* 1997). The human IL-6 gene encodes a polypeptide precursor of 212 aa which is cleaved and secreted as a 184 aa mature glycoprotein of between 21-28 kDa in mass, the range in mass is due to various levels of glycosylation (van Snick *et al* 1988).

Unlike GM-CSF and IL-3, IL-6 can be detected in serum and other body fluids, e.g. in synovial fluids of patients with arthritis. IL-6 can be expressed by a number of cell types including; monocytes/macrophages, stromal cells, fibroblast, endothelial cells and keratinocytes (Kupper *et al* 1989, Richards *et al* 1991). IL-6 expression can be further induced upon stimulation with LPS, phorbol esters and the cytokines IL-1 and TNF (Zhang *et al* 1990) whereas, IL-4 has been observed to act as potent inhibitors of IL-6 production (Denizot *et al* 1999).

In-vitro Musashi and colleagues have shown that IL-6, similar to IL-11 and LIF, enhances IL-3, IL-4 and SCF dependant proliferation of primitive blast cell colonies in methylcellulose cultures (Musashi *et al* 1991a, b). This activity is thought to be due to the growth factors shortening the time the cell spends in Go, and this observation lead to the inclusion of IL-6 in stem cell expansion protocols. Indeed, Bodine *et al* observed that IL-6 in combination with SCF was capable of stimulating the expansion of CFU-S stem cells and maintaining a significant amount of

repopulating ability after 6 days in liquid culture (Bodine *et al* 1994). Furthermore, Sui *et al* demonstrated that stimulation of gp130 by IL-6 and soluble IL6R, resulted in superior *ex-vivo* expansion of primitive human haemopoietic progenitor cells when compared to IL-6 alone (Sui *et al* 1995). More recently a fusion protein of IL6 and IL-6R was observed to be fully active at concentrations of 100-1000 fold less compared to that of unlinked IL-6 and IL6R (Fischer *et al* 1997) in expansion protocols (Chebath *et al* 1997). Other reports have indicated that IL-6 is involved in the development of erythroid, megakaryocytic, myeloid and lymphoid lineages (Kimura *et al* 1990, Hirano *et al* 1996, Sui *et al* 1999). Indeed, Sui *et al* also demonstrated that soluble IL-6R and IL-6 in combination with SCF is involved in megakaryopoiesis and this activity is independent of TPO induced megakaryocyte production (Sui *et al* 1999). The IL-6/sIL-6R complex mimics not only the activity of IL-6 on cells expressing gp130 but also the other members of the IL-6 family of cytokines. For instance, pluripotentiality of ES cells can be maintained by simultaneous addition of IL-6 and sIL-6R but not IL-6 or sIL-6R alone (Yoshida *et al* 1994).

Interleukin-6 exerts these various functions through its receptor which consists of two sub-units, the ligand binding α sub-unit which has a mass of 80 kDa and the signalling gp130 sub-unit which is 130 kDa and both belong to the type I cytokine receptor family. The gp130-signalling sub-unit is also shared with IL-6, LIF, Oncostatin M and CNTF and this results in these cytokines sharing partly overlapping activities. Although gp130 is widely expressed (Saito *et al* 1992) the ligand specific receptor components display a more limited expression, suggesting that cellular responsiveness is largely determined by the regulated expression of the ligand specific receptor chains. The IL-6Rα sub-unit has a short cytoplasmic domain of 82 amino acids and this domain is not needed for signalling as indicated by IL6Rα truncation studies (Taga *et al* 1989). The finding that IL-6α sub-unit exists as a membrane associated and a soluble form indicated that maybe these different isoforms regulate different functions (Narazaki *et al* 1993). Indeed, upon isolation of $CD34^+$ cells it was observed that these could be subdivided into IL-6R expressing and non-expressing cells however, both populations express gp130. It was demonstrated that IL-6Rα expressing cells can be stimulated to form granulocyte macrophage colonies whereas, IL-6Rα negative cells upon stimulation with IL-6 and soluble IL6Rα form

various types of colonies including erythroid bursts, granulocyte macrophage colonies, megakaryocytes and mixed colonies (Sui *et al* 1999). This phenomenon of sIL-6 and sIL6Rα inducing signalling through cells that express gp130 is referred to as transignalling, and as observed by Hirano *et al* it allows cells that do not express IL-6Rα sub-units to respond to IL-6 (Hirano *et al* 1997). The IL-6Rα chain exhibits low affinity for IL-6 however, upon stimulation of cells with IL-6 a high affinity receptor was identified which contained gp130. The high affinity IL-6 receptor has been observed to consist of a hexameric complex containing two IL-6 molecules, two IL-6 alpha sub-units and a homodimer of gp130 (Baumann *et al* 1996). McKinstry *et al* demonstrated that the number of IL-6R on haemopoietic progenitors cells increases significantly with the maturation of these cells (McKinstry *et al* 1997).

IL-6 null mice exhibit a severe impairment in antibody production and acute phase protein production following viral infection and mineral oil injection and they also have a decreased ability to deal with bacterial infections (Kopf *et al* 1994, Dalrymple *et al* 1995). Furthermore, another group examining IL-6 null mice observed that there was a reduced level of leukocyte recruitment, which they suggested was due to the reduced ability of these cells to produce chemokines (Romano *et al* 1997). The various functions of the interleukin-6 family of cytokines on the haemopoietic and lymphoid cell systems, and their ability to function extensively outside these systems is thought to be due to the ubiquitous expression of the gp130 sub-unit (Saito *et al* 1992).

1.7.3.2 Interleukin-11 (IL-11)

An activity that was observed in conditioned media from a primate cell line, PU34 was reported to support the proliferation of an IL-6 dependent plasma cell line. Subsequent studies demonstrated this activity was due to another gp130 signalling cytokine namely IL-11 (Paul *et al* 1990). IL-11 like all the gp130 signalling cytokines is pleiotropic and displays diverse effects such as stimulation of myeloid, erythroid and megakaryocyte differentiation, modulation of macrophage and T cell inflammatory functions and induction of acute phase response proteins, such as fibrinogen and C reactive protein (Baumann 1991). The coding region of Interleukin-11 (IL-11) predicts a polypeptide of 199 amino acids in length which contains a 21 amino acid signal peptide and upon cleavage produces a secreted protein of IL-11 that

has a molecular mass of 19 kDa. Human and murine IL-11 share 88% identity at the primary amino acid level (Morris *et al* 1996), and interestingly IL-11 is unlike the other human gp130 proteins in that it does not have any cysteine residues in its primary aa sequence and has no glycosylation sites.

IL-11 has been detected in fibroblasts from various tissues and can also be produced by bone marrow stromal cells, chondrocytes and osteoblasts and cytokines such as IL-1α, TNF-α, TGF-β and EGF can further induce these cells to produce IL-11 (Elias *et al* 1994a , b).

In the context of the haemopoietic system, Musashi *et al* observed that IL-11 could induce the formation of blast cell colonies *in-vitro* but only in combination with other early acting growth factors such as IL-3, IL-4 or SCF (Musashi *et al* 1991a, b). IL-11 in combination with SCF or IL-3, not only maintains the levels of primitive progenitors in liquid culture it also promotes their growth in methylcellulose (Neben *et al* 1994). IL-11 like IL-6, in the presence of SCF, can also induce primitive quiescent stem cells to exit Go and enter the G1/S phase of the cell cycle and this function has been exploited by several groups in *ex-vivo* expansion protocols (Neben *et al* 1994, Holyoake *et al* 1996). As well as displaying activities on primitive parts of the haemopoietic system, IL-11 alone has been observed to have direct effects on more mature cells. Weich *et al* observed that IL-11 can not only enhance the formation of megakaryocyte colonies (Weich *et al* 1997), it can also induce the differentiation and maturation of megakaryocytes and their precursors in combination with IL-3. *In-vivo* administration of rhIL-11 was observed to increase the number of platelets in animals (Neben *et al* 1993) and more importantly in humans and myelo-suppressed patients (Tepler *et al* 1996), further suggesting the role for IL-11 in megakaryopoiesis. These observations lead to the approval of rhIL-11 as a treatment for chemotherapy induced thrombocytopenia (Gordon 1996). The megakaryocyte lineage is not the only lineage that IL-11 is involved in, it has also been observed that IL-11 plays a role in erythropoiesis as IL-11 in combination with SCF and EPO can stimulate the growth BFU-E and CFU-E colonies (Quesniaux *et al* 1992, Lemoli *et al* 1993, Rodriguez *et al* 1995). The administration if IL-11 to mice resulted in an increase in the number of CFU-GM progenitors within the bone marrow and an increase in the cycling rate of these progenitors and that of the CFU-GEMM progenitors (Hangoc *et al* 1993).

Similar to the IL-6 receptor the IL-11 receptor consists of two sub-units. The ligand specific non-signalling sub-unit IL-11Rα, which can exist in a membrane, bound form or a soluble form, and the signal transducing sub-unit gp130 (Taga *et al* 1997). The IL-11Rα sub-unit consists of an extracellular domain, which contains two N linked glycosylation sites, a transmembrane domain and a small non-signalling cytoplasmic tail (Hilton *et al* 1994). Studies that knockout the IL-11Rα sub-unit have indicated that these animals are healthy and have normal levels of peripheral blood lymphocytes, erythrocytes, platelets, multipotential stem cells (CFU-S) and committed progenitors (CFU-GM, BFU-E and CFU-Meg) (Nandurkar *et al* 1997). Thus suggesting that IL-11 is not essential for regulating haemopoiesis and in its absence its functions are replaced by other growth factors. A further study Robb *et al* observed a similar non-effect in haemopoiesis however, these mice did have an obvious phenotype in that the removal of the IL-11Rα sub-unit renders the female mice infertile (Robb *et al* 1998).

1.7.3.3 Leukaemia Inhibitory Factor (LIF)

Leukaemia inhibitory factor (LIF) was previously known as differentiation inducing factor (D-factor or DIF) and macrophage/granulocyte inducer type 2 (MGI-2) (Tomida *et al* 1984, Hilton *et al* 1988). LIF, like the other gp130 cytokines, is multifunctional and has been observed to inhibit the differentiation of embryonic stem cells, promote the survival and proliferation of primitive haemopoietic precursors and primordial germ cells; induce the expression of acute phase proteins; and is involved in adipocyte differentiation, bone formation and survival of neuronal and muscle cells (Piquet-Pellorce *et al* 1994, Hamilton *et al* 1993, Marshall *et al* 1994, Thaler *et al* 1994, Austin *et al* 1992). LIF is a 180 amino acid glycoprotein whose molecular weight ranges from 38-67 kDa and this heterogeneity has been ascribed to extensive glycosylation (Gough *et al* 1989).

Although LIF is almost undetectable *in-vivo* it has been reported to be expressed by a number of cell types but only after induction with various stimuli. Indeed, the expression of LIF in human bone marrow stromal cells can be induced by phorbol esters, LPS, epidermal growth factor, interleukin-1α and β, TNF, TGF-β and SCF but

not by M-CSF, G-CSF and GM-CSF (Lorgeot *et al* 1997, Gollner *et al* 1999). LIF was originally defined by its ability to induce monocyte differentiation and suppress the differentiation of the murine monocytic leukaemia cell line M1 (Gearing *et al* 1987). On its own LIF has little or no effect on the proliferation or differentiation of primitive haemopoietic progenitors however, in combination with IL-3 or M-CSF, LIF can enhance the proliferation of blast-CFCs, megakaryocyte colonies and CFU-M colonies (Metcalf *et al* 1988, Warren *et al* 1993, Keller *et al* 1996). Furthermore, LIF acts in combination with IL-3, GM-CSF, M-CSF and SCF to promote the colony formation of partially purified lineage negative (Lin^-) bone marrow progenitors (Keller *et al* 1996). However, the most interesting function of LIF is its ability to prevent differentiation commitment of murine embryonic stem cells (ES). These ES cells are normally totipotent and can give rise to all tissues of a mature mouse when injected into the blastocyst, however, in the absence of LIF these ES cells differentiate and lose their totipotency. This activity of LIF in particular has allowed the development of ES based methods such as those used in the production of transgenic and knockout animals (Capecchi 1994). Interestingly, this ability of LIF to inhibit the differentiation has also been observed for some of the other gp 130 signalling molecules such as IL-6, IL-11 and CNTF (Yoshida *et al* 1994).

The receptor for the cytokine leukaemia inhibitory factor (LIF) comprises of a transmembrane protein with low affinity for the cytokine known as gp190 and the gp 130 signal transducing chain (Taga *et al* 1997). Upon LIF binding to gp 190, gp 130 can now interact with this complex and produces a fully functional signalling complex. OSM, CNTF and CT-1 also use gp190 (LIFR) as part of their high affinity receptor complexes. Both sub-units of the LIFR are members of the haemopoietin receptor family however, unlike IL-6 and IL-11R alpha sub-units the low affinity LIFRα chain contains 2 haemopoietin domains separated by an immunoglobulin domain and three fibronectin domains. Recent studies have elucidated that the membrane distal haemopoietin domain along with the Ig domain is essential for LIF binding (Taupin *et al* 1999). Similar to other members of the gp 130 cytokine family, the LIF receptor also has a soluble isoform (Layton *et al* 1992). On examining cross species reactivity it can be observed that murine LIF does not bind to human LIFR whereas, human LIF can bind to murine LIFRα and interestingly human LIF has a higher affinity for murine LIFR complex than murine LIF.

LIF null mice were produced to examine the *in-vivo* role of LIF, these null mice initially appeared to develop and behave normally however, upon further examination they were shown to have a reduced number of stem and progenitor cells in their spleens and bone marrow. Furthermore, it was observed that female mice are unable to produce pups, this was due to the inability of the blastocyst to implant into the uterus. Other LIF null mice have been reported and these mice have been observed to reduce levels of CFU-S stem cells (Escary *et al* 1993). These various reports indicate that LIF is involved in regulating differentiation, haemopoiesis and also plays a role in implantation of the developing blastocyst.

1.7.4 Homo-Dimerising Signalling Growth Factors

1.7.4.1 Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte colony stimulating factor (G-CSF) is a growth factor that acts on the neutrophil lineage to stimulate the proliferation of committed progenitor cells and functionally activates mature neutrophils. The human gene is located on chromosome 17 whereas, the murine gene is clustered on chromosome 11 with the genes for GM-CSF and IL-3 (Nagata *et al* 1989, Platzer 1989).

The G-CSF protein is approximately 18.6 kDa in size and can be produced by a variety of cell types including; macrophages, fibroblasts, endothelial cells and stromal cells, and this expression is further enhanced by IL-1, LPS and phorbol esters (Kothari *et al* 1995).

In-vitro G-CSF has been observed to induce the formation of neutrophilic colonies from the CFU-G progenitors, and enhance the formation of CFU-GM colonies but only at high concentrations (Metcalf and Nicola 1993). In synergy with other growth factors G-CSF can stimulate the survival and proliferation of human myeloid and erythroid progenitor cells (McNiece *et al* 1991). Furthermore, G-CSF can enhance the differentiation and activation of mature neutrophils it also primes these cells to release cytokines and increases their cytotoxicity (Begley 1986).

G-CSF exerts these activities through a single, high affinity, specific receptor which has an approximate mass of 140 kDa and is a member of the haemopoietin receptor family or the cytokine receptor type I superfamily (Demetri and Griffin 1991).

The *in-vivo* role of G-CSF was investigated by Lieshcke *et al* who produced mice that were homozygous for a null mutation of G-CSF. These mice are fertile, viable and have a 70% reduction in the numbers of circulating neutrophils. This indicated that G-CSF is an important regulator of neutrophil production under steady state conditions and emergency situations. Interestingly, these mice also were observed to have a reduced level of progenitors from all lineages suggesting that G-CSF may play a direct role in regulating progenitor levels (Lieshcke *et al* 1994). Indeed, G-CSF and SCF have been observed to enhance the formation of progenitor cells of multiple lineages in developing blast cell colonies (Metcalf *et al* 1991a) and this action may also explain the elevation of multiple lineage elevation of stem and progenitor cells in the blood after G-CSF administration (Roberts and Metcalf 1994). Alternatively, the loss of lineage progenitors may be linked to the decrease in neutrophil levels, as it is possible that neutrophils produce growth factors that are needed for the survival and/or the proliferation of these progenitors.

Numerous reports indicating that G-CSF is the major growth factor involved in neutrophil differentiation lead to the analysis of recombinant G-CSF as a candidate therapy of the neutropenia associated with chemotherapy and radiotherapy. Indeed, G-CSF or Filgrastim as it is clinically known has been observed to decrease the incidence, severity and duration of neutropenia and this has allowed the increase in dose of certain chemotherapy regimes. A further clinical utility of G-CSF was discovered when G-CSF alone or in combination with chemotherapy or chemokines was reported to mobilise progenitors to the peripheral blood. Interestingly these mobilised progenitor cells were observed to be more effective than bone marrow in accelerating platelet and neutrophil recovery in lymphoma patients (Schmitz *et al* 1996).

1.7.4.2 Thrombopoietin (TPO)

Thrombopoietin affects all levels of megakaryocyte development, it not only stimulates haemopoietic stem cells into cycle, it can also support the proliferation of

multipotent and committed megakaryocytic progenitors and induces the expression of various molecules that are needed for platelet formation (Ku *et al* 1996, Broudy *et al* 1995, Papayannopoulou 1996 a, b). Thrombopoietin (TPO) was cloned by a number of groups and was observed to be the ligand for the *c-mpl* receptor. Thrombopoietin mRNA has been detected in the liver, the kidney and to a lesser extent in the bone marrow and spleen (Kuter *et al* 1994, Kaushansky *et al* 1995).

TPO functions primarily as a differentiation factor with limited ability to promote CFU-Meg formation however, in combination with SCF or IL-3, TPO can enhance the formation of these colonies in semisolid media (Broudy *et al* 1995). Further evidence for the role of TPO in megakaryopoiesis was observed *in-vivo*, as the administration of TPO to various animals and humans was shown to increase the number of bone marrow megakaryocytes and peripheral blood platelets (Harker *et al* 1996). As well as TPOs activity on the megakaryocyte lineage, TPO has also been observed to expand cell numbers in mouse long-term cultures and these cells can competitively repopulate a lethally irradiated recipient (Yagi *et al* 1999). Furthermore, the numbers of granulocyte macrophage progenitors (Kaushansky *et al* 1996) and neutrophils is increased upon TPO administration (Sawai *et al* 2000). *In-vitro* in combination with erythropoietin TPO can stimulate the growth of erythroid progenitor cells (Kaushansky *et al* 1995).

These activities of TPO are mediated through its receptor *c-mpl*, which is a member of the cytokine receptor type I superfamily of receptors and requires homo-dimerisation for activation (Bazan 1990). The cytoplasmic domain of *Mpl* is 121 amino acid in length and contains two membrane proximal motifs (box 1 and box 2), these domains are conserved among most members of the cytokine receptor superfamily and are critical for receptor function and signalling via JAKs (Drachman *et al* 1997). A more recent study also provided evidence that TPO is involved in early haemopoietic events, this group isolated early haemopoietic stem cells and further subdivided this population by the expression of *c-mpl*. Upon examining the repopulating ability of these cells it was observed that the *c-mpl*$^{+}$ population essentially contained all of the haemopoietic activity as assessed at 24 week (Solar *et al* 1998).

Although TPOs main function is in the megakaryocyte lineage the haemopoietic defects in mice lacking TPO or its receptor are not restricted solely to cells of the megakaryocyte lineage. *In-vitro* assays have revealed that these TPO null mice display a modest effect on multi-lineages within the haemopoietic system, as indicated by the decrease in the number of CFU-Meg, CFU-GM and BFU-E progenitor cells (Alexander *et al* 1996, Gurney *et al* 1994). Examination of *c-mpl-/-* mice indicated that they have almost no CFU-S stem cells and a reduced level of blast colony forming cells further suggesting a role for TPO in early haemopoiesis as well as regulating megakaryopoiesis (Kimura *et al* 1998).

Platelets are necessary for blood clotting and when numbers are very low individuals are at risk of death from haemorrhage. *In-vivo* data has suggested that the *Mpl* ligand can ameliorate the thrombocytopenia associated with chemotherapy and clinical trials. Indeed, the administration of a pegylated megakaryocyte growth and development factor (PEG-MGDF), which consists of the N terminal region of TPO conjugated to polyethylene glycol, reduces the time the patient is thrombocytopenic and also induces leukocyte and erythrocyte recovery, further suggesting a role for TPO as an adjuvant in transplantation, chemotherapy or radiotherapy. However, care must be taken as TPO receptor *c-mpl* is expressed in some disorders such as acute myeloid leukaemia (AML), and cells from these patients have been observed to proliferate in response to TPO (Matsumura *et al* 1995, Bouscary *et al* 1995).

1.7.4.3 Erythropoietin (Epo)

Erythropoietin promotes the growth, differentiation and survival of erythroid progenitors and is essential for the terminal stages of erythrocyte development. EPO is an approximately 30 kDa glycoprotein and animal studies have indicated that EPO is primarily produced in the kidney during adult life and in the liver in fetal development (Krantz 1991). Further evidence for the kidney being the primary source of EPO in humans was observed as individuals with renal disease are generally anaemic and this anaemia can be reversed by the administration of EPO. EPO has been observed to be produced by murine bone marrow macrophages (Rich *et al* 1982) however, a more recent report indicated that EPO could not be detected in CFU-GM colonies derived from human bone marrow and human peripheral blood macrophages (Stpoka *et al* 1998).

EPO acts mainly on the erythroid progenitor cells, it prevents apoptosis, stimulates proliferation and induces the differentiation of these cells into mature erythrocytes (Kirby *et al* 1996, Liboi *et al* 1993, Kelley *et al* 1993). In addition to erythropoietic activities, EPO has been observed to be involved in the megakaryocyte lineage, and can increase the production of CFU-Meg progenitors from $CD34^+$ bone marrow cells in conjunction with TPO (Papayannopoulou *et al* 1996a). Further evidence for EPOs role in the megakaryocyte lineage in mice was indicated by the increase in platelets and their precursor, megakaryocytes, after EPO administration (Tsukada *et al* 1990). Evidence to suggest that EPO plays a role out with the haemopoietic system was described by Carlini *et al*, they observed that EPO could induce endothelial cell proliferation and protect these cells from LPS induced apoptosis (Carlini *et al* 1999). Furthermore, a previous report also indicated that EPO receptors are expressed in human umbilical vein endothelial cells (HUVEC) (Anagnostou *et al* 1990, 1994) and neurones (Morishita *et al* 1997).

The ability of erythroid cells to respond to EPO is due to their expression of the EPO receptor. The EPO-R is a member of the cytokine receptor type I superfamily and like most of its members require homo-dimerisation for activation (Livnah *et al* 1996). Further evidence for the need for homo-dimerisation for activation of EPO signalling was observed by Schneider *et al*, they indicated that monoclonal antibodies could mimic the homo-dimerisation of the EPO-R and induce proliferation and differentiation of erythroid precursors (Schneider *et al* 1997). The initial steps in the signalling transduction process upon activation of the EPO-R are the activation of JAKs (JAK2) and STAT phosphorylation (STAT5) and this is similar to the other members of the cytokine receptor type I superfamily (Wojchowski *et al* 1999).

The *in-vitro* role of EPO in erythropoiesis was confirmed *in-vivo* as mice lacking the EPO gene or its receptor fail to develop beyond day 13 in utero, due to failure of erythropoiesis at the CFU-E stage in the fetal liver. These mice die of a severe anaemia due to the absence of red blood cells, thus indicating that EPO is crucial for survival, proliferation and differentiation of the late committed progenitors (CFU-E) but not the early progenitors (BFU-E) (Wu *et al* 1995). Further examination of EPO and EPOR null embryos have indicated that these embryos also have a heart defect

and therefore, it seems that EPO signalling system may be involved in heart morphogenesis (Wu *et al* 1999).

The observation that EPO is essential for the terminal differentiation of red blood cells has lead to it being used as a therapy in a number of conditions that are associated with blood loss. Indeed, EPO is used to treat anaemia in renal failure, cancer and HIV patients. It also has also been used to increase platelet number in patients with chronic liver disease (Goodnough *et al* 1997).

1.8 Growth Factor Signal Transduction

1.8.1 Protein Tyrosine Kinase Receptor Signalling

The SCF receptor *c-kit* is a tyrosine kinase receptor (RTK) which is closely related to the receptors for platelet derived growth factor, macrophage colony stimulating factor and Flt ligand. As there is a plethora of data on signalling pathways activated by the numerous tyrosine kinase receptors it was decided to provide a brief overview of the role of *c-kit*/SCF signalling as an example of how intricate and intertwined the protein tyrosine kinase signalling pathways are.

The *c-kit* receptor contains five Ig like domains in its extracellular domain the first, three of which are involved in SCF binding. The fourth Ig domain may play a role in receptor dimerisation and the function of the 5th Ig domain has yet to be discovered. The organisation of the cytoplasmic domain of *c-kit* is similar to *c-fms*, in that both cytoplasmic domains contain a catalytic domain, which is separated by a kinase insert domain. The first catalytic domain contains the ATP binding site and the second site has potential autophosphorylation sites. Before examining the signalling pathways it is worth realising that there are two isoforms of SCF, a soluble and a membrane associated form, and that each has a different effect on the tyrosine kinase activity of *c-kit*. The membrane anchored form induces a prolonged autophosphorylation of *c-kit* compared to the soluble SCF, and this may be due to a slower rate of down regulation via receptor internalisation (Miyazawa *et al* 1995).

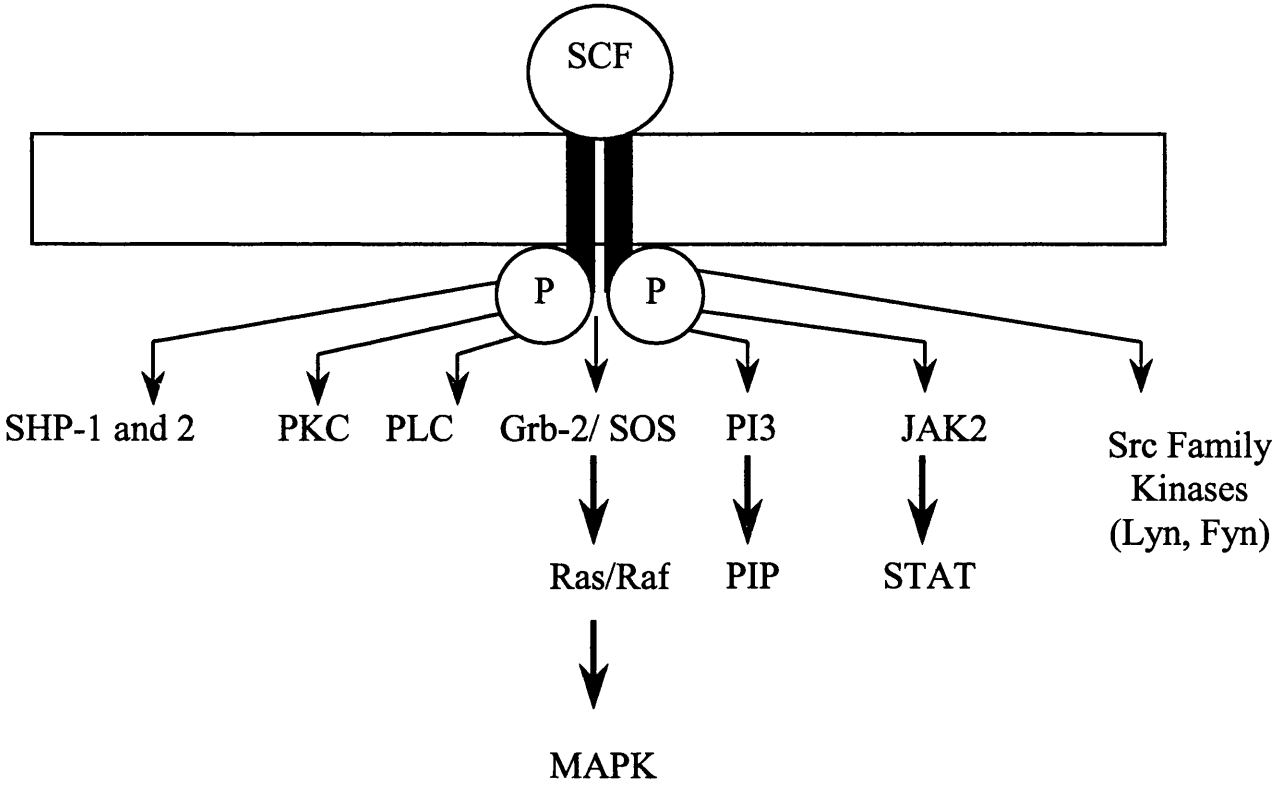


Fig 1.3 An overview of tyrosine kinase receptor signalling pathways

Brief summary of the multiple signalling pathways activated by SCF. SCF induces homodimerisation and auto-phosphorylation of *c-kit* on various tyrosine residues. Various proteins which contain SH2 domains bind to these phosphotyrosines and lead to the recruitment and activation of a number of other proteins e.g. src family members, PI3 K, Grb-2, Ras, Raf-1, MAP kinases, JAKs and STATs. Interaction between different components of these pathways occurs at multiple points and the negative regulators such as SHP-1, 2 and PKC play a role in controlling the signalling pathways.

SCF activates multiple signalling components. Upon ligand binding auto phosphorylation of various tyrosine residues occurs within the *c-kit* molecule and this allows various signalling adapter molecules to interact with these phosphotyrosine via their SH2 domains, such as Crkl and c-Cbl, (Sattler *et al* 1997). Indeed, a number of kinases have been observed to bind to these phosphotyrosines on the *c-kit* receptor such as PI-3 kinase, JAK-2 and members of the src family of kinases such as *lyn, c-Src* and *Fyn* (Lev *et al* 1992, Serve *et al* 1994,Weiler *et al* 1996, Deberry *et al* 1997, Blume-Jensen *et al* 1994, Linnekin *et al* 1997).

Through the actions of these kinases SCF can activate signalling pathways such as the Ras-RAF-MAP kinase cascade and the JAK/STAT pathway (Tsai *et al* 1993). These various SCF stimulated pathways need to be controlled, and this may be down to negative regulators such as SHP-1. SHP-1 and SHP-2 are SH2 containing protein tyrosine phosphatases that have been observed to act as negative regulators of SCF activity in haemopoietic cells (Kozlowski *et al* 1998). Although it may be easier to examine each of these pathways individually one needs to remember that many of these signalling components can interact with multiple pathways induced upon SCF / *c-kit* interaction and therefore, the end results of *c-kit* activation is a result of various signalling pathways.

1.8.2 Glycoprotein 130 (gp130) Signalling

The first step in signalling by members of the IL-6 cytokine family is the specific binding of the particular cytokine to the soluble or transmembrane receptor α sub-unit. The alpha sub-unit is inert in terms of signalling and requires the interaction with the gp130 sub-unit to produce a signal. Ligand binding stimulates homo or hetero dimerisation of gp130 (Timmerman *et al* 2000) and although gp130 possesses no intrinsic tyrosine kinase activity, the dimerisation of gp130 leads to the phosphorylation of gp130. This phosphorylation of gp130 occurs through the activation of a number of the Janus kinase (JAK) family members e.g. JAK1, JAK2 and TYK which have been observed to be associated with gp130 via the membrane proximal box 1 motif in the cytoplasmic domain of gp130 (Tanner *et al* 1995). The tyrosine phosphorylation of gp130 provides a docking site for the SH2 containing proteins such as STAT3 and PI-3 kinase (Zhong *et al* 1994, Minami *et al* 1996, Chen

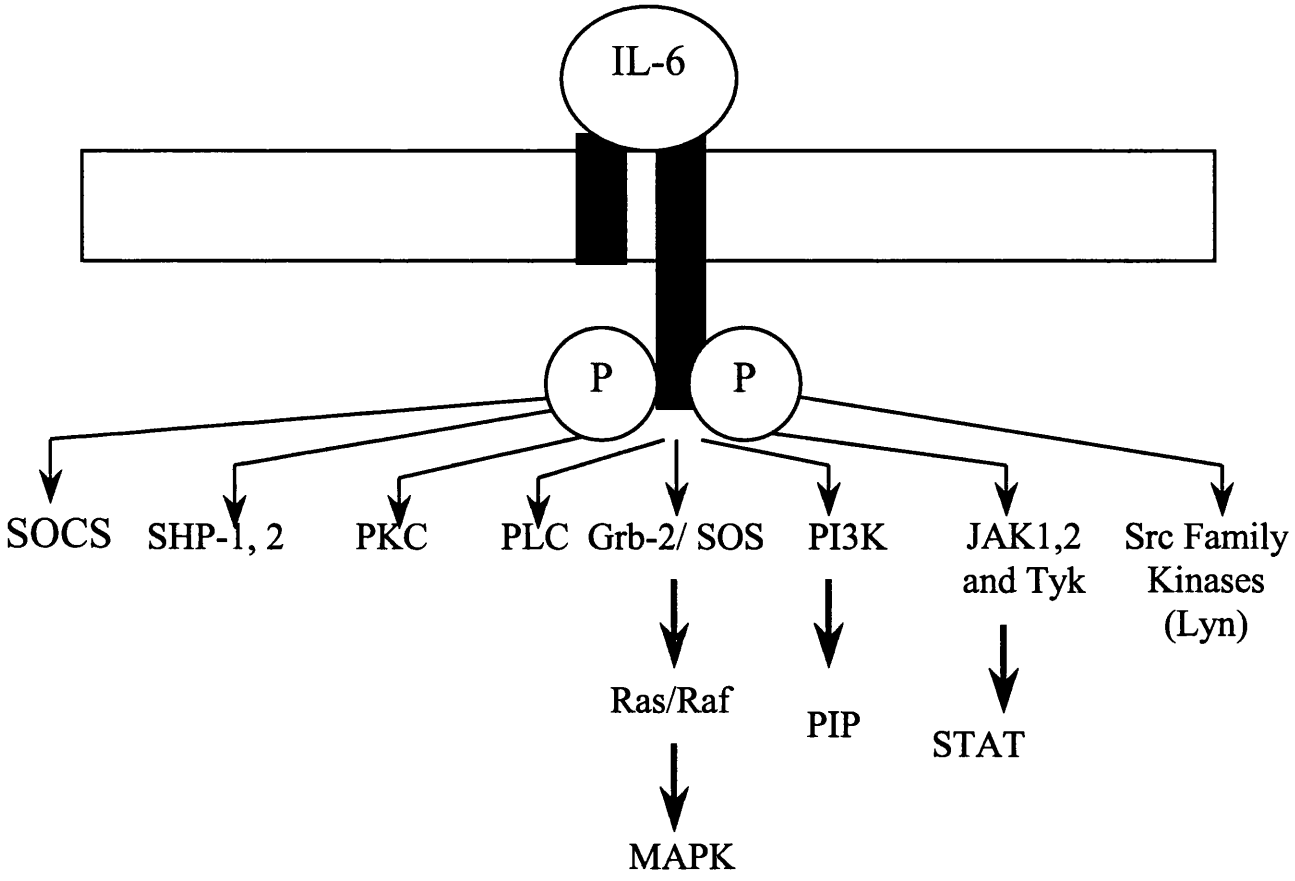


Fig 1.4 Gp 130 signalling pathways

Brief summary of the multiple signalling pathways activated by gp 130 family members e.g. IL-6. IL-6 induces heterodimerisationof the IL-6α and gp130 receptor sub-units, this in turn leads to the phosphorylation of the gp130 sub-unit by associated tyrosine kinases. Various proteins which contain SH2 domains bind to these phosphotyrosines and lead to the activation of src family members, PI3 K,the Ras-Raf-MAP kinase cascade and the JAK/STAT pathway. Interaction between different components of these pathways occurs at multiple points and the negative regulators such as SHP-1 and 2 and PKC play a role in controlling the signalling pathways. Interaction between different components of these pathways occur at multiple points and the negative regulators such as SHP-1 and 2, PKC and SOCS play a role in controlling the signalling pathways.

et al 1999). Besides using the JAK/STAT signalling pathway, several lines of evidence have been observed that suggest that gp130 stimulation can activate the RAS/MAPK signalling pathway. Indeed, IL-6 can increase the proportion of GTP-bound RAS compared to GDP-bound RAS (Nakafuku *et al* 1993), c-Raf-1 a serine threonine kinase known to interact with RAS is also activated by tyrosine phosphorylation (Boulton *et al* 1994). Furthermore, mitogen activated protein kinase (MAPK) becomes hyperphosphorylated thus activating its serine / threonine kinase activity that can then induce phosphorylation of NF-IL6 that can lead to transcription (Nakajima *et al* 1993).

1.8.3 Beta Common (βc) Sub-Unit Signalling

The mechanism of activation of the GM-CSF/ IL-3/ IL-5 receptor system exhibits features that are similar to other members of the cytokine superfamily. Indeed, the βc sub-unit is functionally analogous to the gp130 and IL2Rγ and the common sub-unit of the IL-4 and IL-13 receptors (Zurawski *et al* 1993, Bagley *et al* 1997).

The receptors for IL-3, GM-CSF and IL-5 all lack any intrinsic tyrosine kinase activity (Watanabe *et al* 1996, Itoh *et al* 1996) however, they can upon stimulation via their respective ligands produce a rapid tyrosine phosphorylation of various cellular proteins, these include PI-3 kinase, Shc and the βc sub-unit itself. This is through the action of a number of kinases, which have been reported to be associated with the βc sub-unit *lyn, fes* and JAK2 (Thompson *et al* 1995, Linnekin *et al* 1995, Brizzi *et al* 1996, Al-Shami *et al* 1997, 1998). JAK-2 is a member of the Janus kinase family (Quelle *et al* 1994) and has been observed to be associated with βc sub-unit via the membrane proximal box 1 motif in the cytoplasmic domain of the cytoplasmic tail of the βc sub-unit. Upon activation, JAK2 phosphorylates the membrane distal region of βc and STAT 5 a member of the signal transducer activated transcription family of DNA binding proteins. Phosphorylation of STAT 5 induces dimerisation, which results in translocation to the nucleus where it is involved in gene transcription (Ihle 1996, Itoh *et al* 1998).

As well as tyrosine phosphorylation of βc allowing the activation of the JAK/ STAT pathway it also allows binding of adapter proteins containing src homology 2 (SH2)

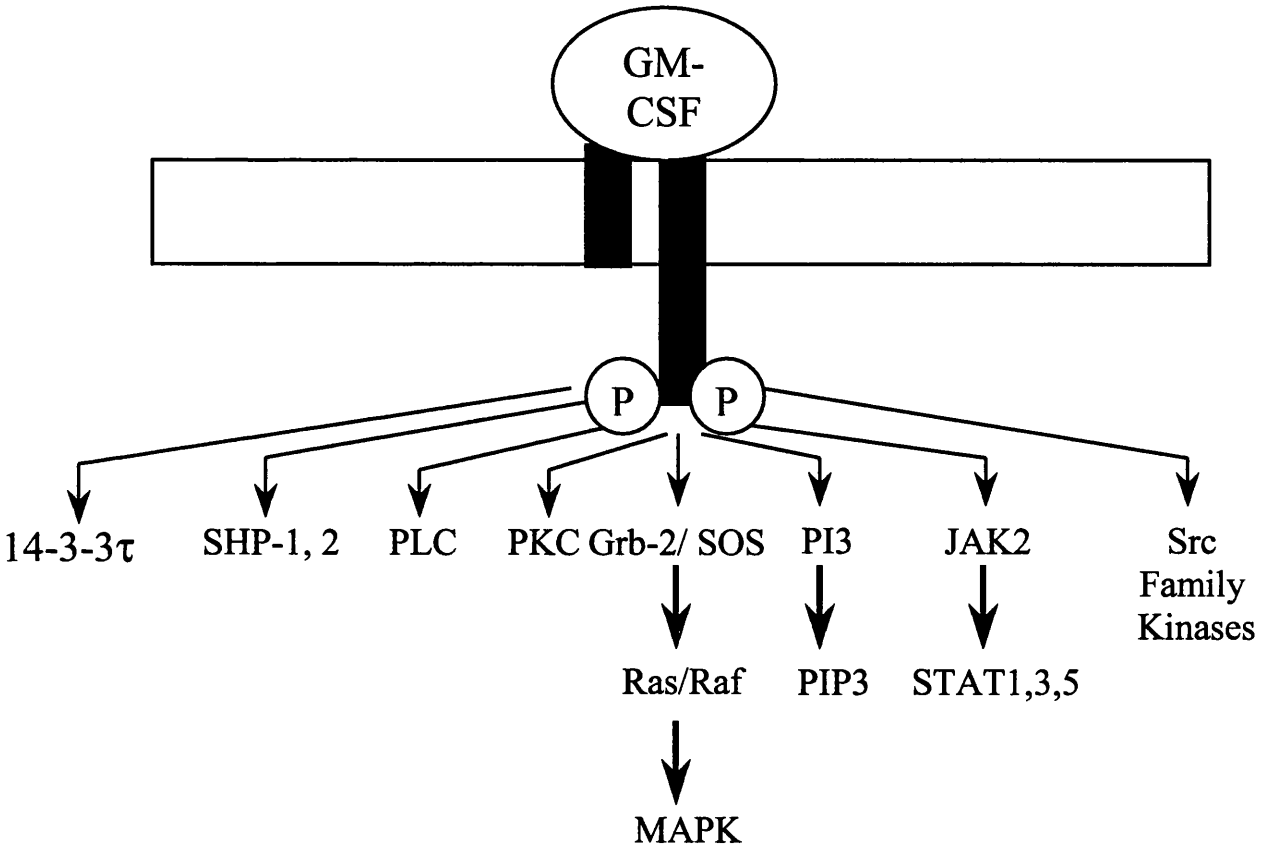


Fig 1.5 Beta common signalling pathways

Brief summary of the multiple signalling pathways activated by GM-CSF. GM-CSF induces heterodimerisation of the α and βc GM-CSF receptor sub-units, this in turn leads to the phosphorylation of the βc sub-unit by associated tyrosine kinases. Various proteins which contain SH2 domains bind to these phosphotyrosines and lead to the activation of src family members, PI3 K, the Ras-Raf-MAP kinase cascade and theJAK/STAT pathway. Interaction between differer components of these pathways occurs at multiple points and the negative regulators such as SHP-1 and 2 and PKC play a role in controlling the signalling pathways. Interaction between different components of these pathways occurs at multiple points and the negative regulators such as SHP-1 and 2 and PKC play a role in controlling the signalling pathways.

domains. Shc is one such example and it has been observed to recruit grb2 (Gale *et al* 1998), the further recruitment of SOS forms a complex, which activates p21 ras nucleotide exchange. This leads to the activation of ras and downstream partners on the mitogen activated protein (MAP) kinase pathway, including Raf (Okuda *et al*1997). Other studies have demonstrated that it is not only the tyrosine phosphorylation that is important for GM-CSF signalling. Indeed, a serine phosphorylation sequence was found in the βc sub-unit that can be serine phosphorylated on activation with GM-CSF and can interact with an adapter protein 14-3-3 ζ therefore, serine phosphorylation may also play a role in regulating the signalling of GM-CSF (Stomski *et al* 1999).

1.9 Growth Inhibitors

As well as positive growth regulators there are also negative growth regulators that may interact with stem and progenitor cells within the bone marrow to control the proliferation of these cells. The major inhibitors of stem cell proliferation include the tetrapeptide (AcSDKP), the pentapeptide (pEEDCK), TNF-α, IFN-γ, MIP-1α and TGF-β (Bonnet *et al* 1995, Frindel and Guigon 1977, Laerum and Maurer 1973, Zhang *et al* 1995, Snoeck *et al* 1994, Graham *et al* 1990, Keller *et al* 1994). A brief description of each of these inhibitors and the part of the stem cell compartment that they exert their inhibitory effect on is outlined below however for further information on these inhibitory molecules see Graham and Wright 1997a, Graham 1997b.

1.9.1 The Tetrapeptide (AcSDKP)

The tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) was initially isolated from fetal calf bone marrow and is now chemically synthesised and known as Goralatide (Lenfant *et al* 1989). The AcSDKP sequence has been found in a number of larger proteins such as Thymosin-β4 (Tβ4) and TNF-α (Grillon *et al* 1990) and has been observed to be present in human plasma from healthy individuals (Ezan *et al* 1994). AcSDKP has been observed to be constitutively produced *in-vitro* by bone marrow cells in murine long-term cultures (Wdzieczak *et al* 1990) and a more recent report indicated that the macrophage is the major AcSDKP producing cell (Li *et al* 1997).

In-vitro AcSDKP can reversibly inhibit the proliferation of CFU-GM, CFU-Meg and BFU-E progenitor cells, it can also inhibit the proliferation of more primitive haemopoietic cells such as HPP-CFC and human LT-CIC. Furthermore, AcSDKP has been observed to inhibit the proliferative response of purified human $CD34^+$ cells to a cocktail of growth factors (Jackson *et al* 1996, Aidoudi *et al* 1998, Robinson *et al* 1992, Bonnet *et al* 1993). Although, the sequence SDKP has been observed in TNF-α both these molecules regulate the proliferation of human $CD34^+$ cells differently (Bonnet *et al* 1995) and unlike TGF-β, TNF-α, MIP-1α and pEEDCK all of which exhibit bi-directional growth activities, AcSDKP has never been observed to exert any positive growth signals.

These inhibitory activities of AcSDKP *in-vitro* were confirmed *in-vivo* as administration of AcSDKP was observed to prevent the recruitment of the colony forming unit-spleen (CFU-S) into S-phase and increase the rate of survival of mice which have been treated with cytosine-arabinoside (Ara-C) (Bogden *et al* 1991). As well as protecting cells from Ara-C, AcSDKP has also been observed to protect stem cells and progenitor cells from various agents such as cyclophosphamide, 5-fluorouracil (5-FU), doxorubicin, ionising radiation and hypertherapy or phototherapy (Bogden *et al* 1991, Aidoudi *et al* 1996, Masse *et al* 1998, Watanabe *et al* 1996, Wierenga *et al* 1997, Coutton *et al* 1994). The inhibitory properties of AcSDKP as well as its inability to inhibit leukaemia cells (Bonnet *et al* 1992) suggested that it may have a therapeutic role as a myeloprotective agent during chemotherapeutic treatment of cancer. This was confirmed as AcSDKP in association with a chemotherapeutic regimen in cancer patients reduces the period of neutropenia (Carde *et al* 1992). However, its use is limited due to its short half-life and this was observed to be due to its breakdown by an enzyme normally associated with regulating blood pressure, angiotensin-converting enzyme (ACE). A potential role for angiotensin converting enzyme (ACE) in the regulation of haemopoiesis was observed as administration of Captopril, an inhibitor of ACE, was observed to increase the plasma concentrations of AcSDKP by 5-6 fold (Azizi *et al* 1996, Rousseau *et al* 1995, Li *et al* 1997). These studies indicate that the tetrapeptide (AcSDKP) displays inhibitory properties on a range of cells in the stem cell compartment from mature progenitor cells to immature progenitor/stem cells.

1.9.2 The Pentapeptide (pEEDCK)

The pentapeptide pGlu-Glu-Asp-Cys-Lys (pEEDCK) is produced in granulocytes, (Paukovitis *et al* 1983) and its sequence has been observed in the alpha sub-units of G-proteins (Pfeilstocker *et al* 1996). pEEDCK has been observed to reversibly inhibit the proliferation of bone marrow progenitor cells and has been reported to protect mice from the myelotoxicity associated with Ara-C (Paukovitis *et al* 1990). Paukovitis also observed that pEEDCK could not only inhibit the formation of CFU-GM colonies, it could also inhibit the proliferation of CFU-S stem cells *in-vivo* (Paukovitis *et al* 1993). Furthermore, the addition of pEEDCK to long term bone marrow cultures inhibits progenitor production from LTC-ICs (Paukovitis *et al* 1995).

Interestingly, the oxidation of the thiol group of pEEDCK leads to the formation of a disulphide bonded dimer which has been observed to increase the production of CFU-GM in long term bone marrow cultures and has no inhibitory activity (Laerum *et al* 1998, Paukovitis *et al* 1995). Activated granulocytes can rapidly oxidise monomeric pEEDCK to the dimeric form *in-vitro* (Paukovitis *et al* 1998) and the administration of $(pEEDCK)_2$ to mice lead to an increase in myeloid progenitors, megakaryocytes, mature leukocytes and platelets (Paukovitis *et al* 1995). The growth promoting and inhibitory activities of this pentapeptide suggest this one molecule alone could control the regulation of stem cell proliferation.

1.9.3 Tumour Necrosis Factor-alpha (TNF-α)

TNF-α is a member of a family that consists of TNF-β or lymphotoxin alpha (LT-α) and lymphotoxin beta (LT-β). Although there are several cell types that produce TNF-α, the main source of the cytokine are the monocytes and macrophages and these cells can be further induced to produce TNF-α by LPS and IL-1. TNF-α is a pleiotropic cytokine and has a number of functions on a variety of cell types; it can induce cytokine expression in T cells, fibroblasts, monocytes and endothelial cells; TNF-α can induce the express of adhesion molecules on endothelial cells and it can also induce the expression of antibodies in B cells and acute phase protein production in hepatocytes.

TNF-α is a bi-directional cytokine as it can stimulate or inhibit cell growth (Backx *et al* 1990). Indeed, TNF-α has been observed to potentiate human progenitor cell growth in conjunction with IL-3 and GM-CSF and inhibit the same cells grown in SCF, M-CSF or G-CSF (Caux *et al* 1990, Rusten *et al* 1994a, b). The inhibitory activity of TNF-α is not restricted to committed progenitors as TNF-α has been observed to inhibit HPP-CFC colony formation and colony formation of primitive haemopoietic cells (Sca-1^+/ Lin^-) (Jacobsen *et al* 1994).

TNF-α exerts these various functions through interaction with, two distinct TNF-α receptors a 55 kDa (TNFR55) and a 75 kDa (TNFR75) both of which exist as soluble forms (Aderka *et al* 1992). TNF-α has been observed to induce distinct functions depending on which receptor it interacts with, indeed upon activation of signalling through TNFR55, TNF-α induces proliferation of fibroblasts and prostaglandin synthesis, whereas, signalling through TNFR75 by TNF-α has been reported to mediate T cell, mononuclear, fibroblast and NK proliferation and induction of GM-CSF in T cell lines (Naume *et al* 1991). Furthermore, TNF-α signalling through TNF-75 (75 kDa TNF-Receptor) has been observed to inhibit primitive cells whereas, the signal through TNF-55 (55 kDa TNF-Receptor) inhibits more committed progenitors isolated from bone marrow from mice and humans (Rusten *et al* 1994b, Jacobsen *et al* 1995b). These observations were confirmed by using specific antibody agonists to the two different receptors and studies examining the different activities of hTNF-α and mTNF-α, i.e. hTNF-α can not bind to TNFR 75 and thus can not inhibit primitive cell proliferation (Rusten 1994b). However, a contradictory report examining TNFR55 null mice observed that primitive haemopoietic stem cells, Sca-1^+ /Lin^- /$ckit^+$ cells, are increased in number and TNF could not inhibit the proliferation of these cells, suggesting that TNFR55 is the major receptor involved in regulation of these HSC (Zhang *et al* 1995). This contrasting data may be due to the use of two different types of primitive progenitors i.e. Sca-1^+ /Lin^- in the previous study compared to Sca-1^+ /Lin^- /$ckit^+$. These studies indicate that signalling via TNF is complex and although each receptor has been observed to signal separately for many TNF responses, Tartaglia proposed that signalling through one of the receptors can only be obtained through the interaction with the other (Tartaglia *et al* 1993).

1.94 Transforming Growth Factor-beta (TGF-β)

Transforming growth factor beta (TGF-β) belongs to a large superfamily of growth factors that all share varying degrees of homology with TGF-β. This family can be subdivided into four groups the TGF-β family, the bone morphogenic protein (BMP) family, the inhibin/activin family, and the MIS (Mullerian inhibiting substance) family. TGF-β(1–3) can all inhibit the growth of epithelial, endothelial, keratinocytes and haemopoietic cells and although TGFβ1 was initially shown to be the most potent isoform, further studies have indicated that TGF-β3 is in fact the most potent isoform (Alexandrow *et al* 1995, Heimark *et al* 1986, Sitnicka *et al* 1996, Hatzfeld *et al* 1996). TGF-β1, 2 and 3 are located on chromosome 19, 1 and 14 in humans and on chromosome 7, 1 and 12 in mice (Fujii *et al* 1986, Guron *et al* 1995). TGF-β expression has been observed to be ubiquitous and it is secreted from the cell of origin in a latent form. This latent form consists of a mature dimer plus two pro-region peptides called latency-associated proteins (LAP) and the removal of this produces an active TGF-β homodimer.

In-vitro TGF-β has been observed to inhibit the formation of CFU-GEMM, CFU-GM and CFU-M colonies in the presence of GM-CSF from human long term bone marrow cultures, and similarly TGF-β also inhibited the formation of these progenitor colonies in the presence of IL-3 in murine long term bone marrow cultures (Ohta *et al* 1987). TGF-β inhibitory properties have also been observed in the erythropoietic and megakaryocytic lineages as TGFβ-1 can inhibit the formation of BFU-E, CFU-E and CFU-Meg colonies. TGF-β has also been observed to induce apoptosis of primitive haemopoietic progenitor cells (Jacobsen *et al* 1995a). TGF-β also displays positive growth properties as Keller *et al* have observed that TGF-β1 can augment GM-CSF supported CFU-GM colony formation and also inhibit the formation of these same colonies that are stimulated by IL-3 (Keller *et al* 1991). The inhibitory or stimulatory activity of TGF-β is determined by the context in which it is acting i.e. the specific nature of the target cells or the presence of growth factors (Keller *et al* 1994).

Apart from TGF-β's ability to inhibit and stimulate haemopoietic stem and progenitor cells it also plays other important roles in the bone marrow microenvironment that may effect the proliferative state of these cells. Indeed, TGF-β1 has been observed to regulate the adhesiveness of cells by increasing the synthesis of extracellular matrix protein expression, control of matrix degrading proteases and protease inhibitors and increased expression of cell surface receptors for cell adhesion such as integrins. It can also regulate the expression of various growth factors and their receptors as indicated by the reciprocal interaction between TGFβ and MIP-1α in bone marrow macrophages (Maltman *et al* 1996). A possible mechanism for TGF-β inhibition or stimulation of cell growth is via the down regulation of various growth factor receptors.e.g. TGFβ can downregulate EGF (Takehara *et al* 1987), SCF, GM-CSF and IL-3 receptors (Jacobsen *et al* 1993) and up regulate the expression of CCR-1, 3, 5, 6 and CXCR-4 on dendritic cells (Sato *et al* 2000).

The *in-vivo* functions of TGFβ-1 were initially examined by injecting mice with TGF-β1. TGFβ-1 administration was observed to inhibit the proliferation of CFU-S and this inhibition was reversible as the growth of these progenitor cells began to recover after 24 hours after the withdrawal of the treatment (Migdalska *et al* 1991). TGFβ-1 was also observed to accelerate the recovery of mice after a dose of 5-FU (Grzegorzewski *et al* 1994) furthermore, TGF-β1 could also inhibit intestinal epithelial cells. These functions of TGF-β1 indicate that it can play an important role in controlling the proliferation of stem and progenitor cells. A further examination of TGF-β1 *in-vivo* function was observed by disrupting the TGF-β1 gene. In doing so it was observed that approximately 50 % of these TGF-β1 -/- embryos died before birth and the other 50 % survived however, these mice died after 2-3 weeks due to a rapid wasting syndrome (Kuilkarni *et al* 1993). Further studies have indicated that the survival of some of the TGF-/- embryos was due to the maternal transfer of TGF-β1 via the placenta (Letterio *et al* 1994). The wasting disorder observed in these TGF-β1 -/- mice is characterised by the infiltration of various tissues such as heart, lung and liver by inflammatory cells and an increase in pro-inflammatory cytokines such as TNF-α and MIP-1α, which eventually leads to cell death and tissue necrosis. However, this can be reversed by the administration of an immuno-suppressing agent, Rapomycin (Borkowski *et al* 1996). A further TGFβ-1 null mice study indicated that the reason for the death of these embryos prior to birth was due to defects in

haemopoiesis and blood vessel formation in the yolk sac (Dickson *et al* 1995). Therefore, these TGF-β1 null mice studies indicate that TGF-β1 has a role to play in early haemopoiesis, vasculogenesis and in inflammation

1.95 Macrophage Inflammatory Protein-1 alpha (MIP-1α)

A more detailed description of MIP-1α and its various activities can be found in section 2.52. Briefly, MIP-1α is a member of the pro-inflammatory CC chemokine family that has been observed to reversibly inhibit the short term repopulating components of the stem cell compartment. Furthermore, it also displays various functions from inflammatory activities on mature cells to inhibition of keratinocytes and stem cell proliferation. MIP-1α or stem cell inhibitor (SCI) as it was previously known has been observed to inhibit the transiently engrafting stem cells such as the CFU-A and CFU-S stem cells (Pragnell *et al* 1988, Lord *et al* 1992). However unlike the previously described inhibitors, it has no inhibitory activity on mature progenitors or on long term repopulating stem cells or other primitive cell types such as HPP-CFC progenitors (Bonnet *et al* 1995, Broxmeyer *et al* 1990, 1999, Graham *et al* 1990, Quesniaux *et al* 1993, Keller *et al* 1994). MIP-1α can protect CFU-S and CFU-A stem cells following treatment with the cytotoxic drug cytosine arabinoside (ARA-C) by preventing these stem cells from entering S phase of the cell cycle (Dunlop *et al* 1992). This ability lead to MIP-1α being examined as a myeloprotective agent in cancer treatment however, contradictory results have been observed on its efficiency to act as a myeloprotective agent (Broxmeyer *et al* 1998, Bernsteain *et al* 1997, Clemons *et al* 1998). Under steady state conditions, it is likely that the majority of haemopoietic function is regulated at the level of the transiently engrafting stem cells with little input from the primitive long term repopulating cells. Therefore, it is likely that the transient engrafting cells, the target of MIP-1α, may be more in need of regular growth control. This may partially explain the relatively restricted pattern of MIP-1α function within the stem cell compartment. Interestingly, MIP-1α null mice have no reduction in their short term repopulating stem cells when compared to wild type animals suggesting that MIP-1α is not essential for the negative regulation of stem cell proliferation. However it may be possible that in the absence of MIP-1α some other growth inhibitor fulfils the inhibitory function of MIP-1α.

CHAPTER 2 : INTRODUCTION

2.1 MIP-1α and the Chemokine Super Family : An Overview

The role of macrophage inflammatory protein-1 alpha (MIP-1α) as a haemopoietic stem cell regulator was initially identified by Graham *et al* in 1990. They purified a reversible inhibitor of CFU-A and CFU-S stem cell proliferation from a murine macrophage cell line J774.2 (Graham *et al* 1990). Sequence analysis demonstrated that this protein, which they referred to as Stem cell Inhibitor (SCI), was indeed identical to a previously described molecule Macrophage inflammatory protein -1 alpha (Wolpe *et al* 1988). MIP-1α is a member of a rapidly expanding family of pro-inflammatory mediators known as chemokines, the various members of which are involved in multiple processes, which include development, haemopoiesis (Graham *et al* 1990), angiogenesis (Streiter *et al* 1995) and regulation of specific leukocyte trafficking (Sozzani *et al* 1997). The chemokine field has recently become the focus of much attention after the major discovery that chemokines and their receptors have a role to play in HIV pathogenesis.

The initial members of the chemokine family were identified by biochemical purification of biological activities in cell supernatants and subsequent cloning. More recently, molecular biologists have identified chemokines by constructing cDNA libraries from subtractive hybridisation (Schall *et al* 1988) and signal sequence trap cloning (Tashiro *et al* 1993) techniques, and screening these libraries against various databases such as expressed sequence tags (EST) databases (Rossi *et al* 1997). This has lead to the expansion of chemokine numbers in recent years, from a handful of proteins, to in excess of 40. The name chemokine was proposed to convey the combined chemoattractant and cytokine properties that had been identified for many of these peptides. Chemokines in general are monomeric and constitute a superfamily of small 7-18 kDa basic, heparin binding, inducible and secreted pro-inflammatory cytokines that can be identified by their sequence homology and the arrangement of conserved cysteine residues (Baggolini *et al* 1994, Rollins *et al* 1997, Zlotnik *et al* 1999). Until recently, the chemokine superfamily was divided up into two subgroups, however, there is now evidence to support the existence of a further two subgroups within the chemokine superfamily. The two major chemokine subgroups are the CXC

and the CC subgroup. The CXC subgroup are so called because the two cysteine residues nearest the N terminus of these proteins are separated by a single amino acid (Baggolini *et al* 1997), whereas, the CC subgroup have their first two cysteines adjacent to one another (see section 2.41, 2.42 and 2.51, 2.52 for a detailed description of two CXC and CC respectively). The two newest subgroups of the chemokine superfamily are the "C" and the "CX_3C" subgroups. The chemokines that belong to these subgroups have either a lone cysteine in the N terminal domain or have three amino acids separating their two N-terminal cysteine residues, and these will be discussed in sections 2.2 and 2.3. (Kennedy *et al* 1995, Kelner *et al* 1994, Pan *et al* 1997, Bazan *et al* 1997). For further information on chemokine nomenclature and recent discoveries the reader is referred to a review by Zlotnik (Zlotnik *et al* 1999).

2.2 The C Superfamily

Lymphotactin (Ltn) was cloned from an activated mouse pro-T cell (Kelner *et al* 1994) and its human homologue single cysteine motif chemokine 1 (SCM-1) was subsequently isolated (Yoshida *et al* 1995). Lymphotactin is similar to members of the CC and CXC super families, however, it only retains the 2^{nd} and 4^{th} cysteines therefore, Ltn can only make one of the two di-sulphide bonds that are characteristic features of the quaternary structure of the chemokines (Kennedy *et al* 1995). In humans there are two homologous genes localised on chromosome 1 that encode two SCM-1 proteins, SCM-1α and SCM-1β, and these differ by only two amino acids (Yoshida *et al* 1996). Interestingly, both the murine and human genes are found on chromosome 1, an unusual location for chemokine genes (Kelner *et al* 1994, Kennedy *et al* 1995). Lymphotactin expression has been detected in activated pro-T cells, $CD8^+$ T cells, NK cells (Hedrick and Zlotnik 1997 a, b), dendritic epidermal T cells (Boismenu *et al* 1996) and mast cells (Rumsaeng *et al* 1997). Although, Ltn can act as a chemotaxin for lymphocytes and NK cells, but not monocytes or neutrophils, the exact role of Ltn is as yet unclear (Kelner *et al* 1994). An orphan receptor first identified by Heiber *et al*, which is now known as XCR1, has been observed to be a functional receptor for Lymphotactin and SCM-1β. Interestingly XCR1 has been observed to be highly expressed in placenta, with low levels in spleen, thymus and peripheral blood cells (Heiber *et al* 1995, Yoshida *et al* 1998). Lymphotactin and SCM-1β are important chemokines not only because of their unusual structure,

expression pattern and chromosomal location, but also because they are one of the growing numbers of chemokines that can induce chemotaxis of lymphocytes (Loetscher *et al* 1996).

2.3 CX_3C Superfamily

One of the more interesting observations in the chemokine field was the identification of an atypical chemokine, fractalkine or neurotactin (Bazan *et al* 1997, Pan *et al* 1997). To date, this is the only member of a fourth chemokine subgroup, which contains the CX_3C fingerprint. This molecule was identified from non haemopoietic cells and has been shown to be a 373 amino acid glycoprotein that carries the chemokine domain on top of an extended mucin like stalk domain. Fractalkine can exist in two forms, either a membrane anchored moiety, as predicted by a stretch of 18 hydrophobic residues making this domain the likely transmembrane domain, or a soluble glycoprotein of 95 kDa. These two forms of fractalkine have different functions, the soluble form has a potent chemoattractant activity for monocytes and T cells (Bazan *et al* 1997, Imai *et al* 1997), whereas, the expression of the membrane bound form, which can be actively induced on primary endothelial cells by IL-1 and TNF-α, promotes leukocyte adhesion. Studies have indicated that the chemotactic activity of fractalkine can be inhibited by pertussis toxin, whereas, the direct adhesion of cells to tethered fractalkine cannot. Thus indicating that signalling through a pertussis toxin sensitive receptor is needed for the chemotactic activity of fractalkine, but not its adhesive function (Imai *et al* 1997, Haskell *et al* 1999). Pan *et al* alternatively cloned Fractalkine as neurotactin in 1997, and its messenger RNA is predominantly expressed in mouse brain. Both groups identified the chromosomal location to be distinct from the majority of other chemokine families, human chromosome 16 (Bazan *et al* 1997) and chromosome 8 in the mouse (Rossi *et al* 1998). However more recently, two CC chemokines genes for monocyte-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) have been shown to be located on chromosome 16 (Imai *et al* 1998, Nomiyama *et al* 1997). The exact function of this fourth chemokine subfamily has yet to be defined however, fractalkine is a versatile molecule that can regulate both cell-cell interactions as the membrane bound form and directed cell migration by the soluble form. A more recent report indicates that fractalkine may well be involved in leukocyte trafficking within the central nervous system (CNS), as expression of both fractalkine and its receptor CX_3CR1 have been

detected in the CNS (Nishiyori *et al* 1998). Interestingly, CX_3CR1 has been observed to be an HIV co receptor in-vitro (Combadiere *et al* 1998), however, the relevance of this *in-vivo* has still to be recognised. Thus, further investigation is needed to uncover the functions of fractalkine and CX_3CR1 *in vivo*.

2.4 The CXC Superfamily

The CXC superfamily consists of at least 15 chemokines and there may be more to be found. Platelet factor-4 (PF4) was the first CXC protein to be characterised. Its sequence was reported in 1977, 10 years before the discovery of IL-8 (Walz *et al* 1977). CXC chemokines with biological activities similar to PF-4 and IL-8 were discovered e.g. GRO-α, β and γ, ENA-78, IP-10 and Mig (For an up to date list of the members of the chemokine families see figure 2.1). The CXC chemokines can be further subdivided into chemokines that contain the motif ELR prior to their first cysteine residue and those that do not contain the ELR motif. Interleukin-8, GRO-α, β, γ, ENA-78, and GCP-2 are a few of the CXC chemokines that contain the ELR motif, and they generally all have the ability to chemoattract and activate neutrophils (Clark-Lewis *et al* 1993 1994, Rollins *et al* 1997), whereas, the non ELR CXC chemokines, IP-10, Mig, SDF-1α /β, I-TAC and BCA-1 have little or no effect on neutrophils and attract activated T lymphocytes (Taub *et al* 1995). The introduction of the ELR motif into PF-4, a non-ELR containing CXC chemokine, enables this chemokine to chemoattract and activate neutrophils, however, this is not always the case, as the introduction of the ELR motif into IP-10, Mig or the CC chemokine MCP-1 has no effect on their activities (Clark-Lewis *et al* 1993). A full discussion of the CXC chemokine members is beyond the scope of this work, therefore, only two CXC chemokines will be discussed in more detail below, IL-8 (an ELR CXC chemokine) and SDF-1 (a non-ELR CXC chemokine). These chemokines are of particular interest as both have activities that are involved haemopoiesis.

2.4.1 Interleukin -8 (IL-8)

The prototypic CXC chemokine IL-8 was purified and originally described as MDNCF (monocyte derived neutrophil chemoattractant factor), (Schroeder *et al* 1987) and NAP-1 (neutrophil activating protein-1) due to its ability to chemoattract

neutrophils *in-vitro* (Walz *et al* 1987). The cDNA corresponding to Interleukin-8 was cloned shortly after by Matsushima *et al* in 1988 (Matsusihima *et al* 1988).

Human IL-8 can be synthesised and secreted by a host of cell types including monocytes/macrophages (Schroeder *et al* 1987, Yoshinumra *et al* 1987), T cells (Gregory *et al* 1988), neutrophils (Streiter *et al* 1990a) and endothelial cells (Streiter *et al* 1989, Utgaard *et al* 1998) and can be further induced by IL-1, TNF-α and LPS. Two distinct forms of human IL-8 have been identified (Van Damme *et al* 1990, Yoshimura *et al* 1989), the most abundant form being the naturally occurring one which is 72 amino acids in length. The alternatively spliced form of IL-8 is 77 amino acids in length and is produced by endothelial cells, and therefore is sometimes referred to as endothelial IL-8. This larger protein is 10 fold less potent at attracting and activating neutrophils *in-vitro* (Gimbrone *et al* 1989). However, *in-vivo* there is no apparent difference in neutrophil attraction or activation by either of these proteins and this is thought to be due to N terminal processing that occurs *in-vivo* and not *in-vitro*.

Although IL-8 is an inflammatory cytokine that is known to function as a neutrophil chemoattractant and activating factor (Baggolini *et al* 1992), it can also act as a chemotaxin for monocytes, NK cells (Morohashi *et al* 1995), T cells (Bacon *et al* 1995) and mast cells (Nilsson *et al* 1999). Other properties attributed to IL-8 include induction of oxidative burst and lysosomal enzyme release in neutrophils (Baggolini *et al* 1992), histamine release by basophils (Dahinden *et al* 1989) and IL-8 may also have a role to play in angiogenesis (Koch *et al* 1992, Strieter *et al* 1995). Upon injection of IL-8 into mice, the entire inflammatory process of neutrophil recruitment can be observed in tissues, and this was initially thought to be due to the diffusion of IL-8 between the pericellular space of the endothelial cells of the blood vessels (Swensson *et al* 1991). However, a more recent study observed that IL-8 produced in tissues was transcytosed across the endothelial cells (EC) and presented at the lumenal surface. Furthermore, this study also suggested that the interaction of IL-8 with the promiscuous chemokine receptor DARC and heparan sulphate were involved in this process (Middleton *et al* 1997).

A role for IL-8 in the haemopoietic system was postulated by Broxmeyer *et al*, who observed that IL-8 could suppress colony formation of mature progenitor cells from

unfractionated bone marrow grown in SCF and GM-CSF (Broxmeyer *et al* 1993). A later study by Graham *et al* using growth conditions that induce the proliferation and differentiation of a more primitive population of cells, transiently engrafting stem cells referred to as colony forming unit-agar CFU-A, indicated that IL-8 could not inhibit the formation of these CFU-A colonies (Graham *et al* 1993). This may indicate that IL-8 like MIP-1α has a restricted inhibitory role in haemopoiesis. A further possible role of IL-8 in haemopoietic system was observed by Laterveer *et al*, who described that IL-8 alone or in combination with SCF could mobilise progenitor cells to the peripheral blood (Laterveer *et al* 1996). A further study by Liu *et al* reported that IL-8 was unable to induce mobilisation of these stem and progenitor cells in G-CSF null mice, and they therefore suggested that IL-8 indirectly mobilises these cells by the possible increase in expression of G-CSF (Liu *et al* 1997). However, a further study by Pruijt *et al* observed that antibodies to the β2 integrin LFA inhibited the mobilisation of progenitor and stem cells in mice injected with IL-8 (Pruijt *et al* 1998). From this observation, they suggested that IL-8 may alter the adhesiveness of these progenitor cells and this may be an important step in mobilisation of these cells to the peripheral blood.

IL-8 has been observed to induce its various activities by binding to two specific receptors; these receptors were originally called IL-8RA and B but are now referred to as CXCR1 and CXCR2. CXCR1 is more selective in respect to ligands than CXCR-2, as CXCR1 can only interact with IL-8 and GCP-2 with high affinity, whereas, CXCR2 has high affinity for IL-8 and all other ELR containing CXC chemokines e.g. growth related oncogene (GRO) proteins, epithelial neutrophil attractant-78 (ENA-78), neutrophil activating protein-2 (NAP-2) and granulocyte chemotactic peptide-2 (GCP-2). Human CXCR1 cDNA was first isolated by Holmes *et al* in 1991, and a year later Lee *et al* isolated the cDNA for the human CXCR2 receptor (Holmes *et al* 1991, Lee *et al* 1992). These receptors are 78 % identical to one another and the genes were found on chromosome 2. Expression of both receptors have been identified on various mature blood cells neutrophils, monocytes/macrophages and on cytokine activated eosinophils, basophils, T lymphocytes, mast cells and dendritic cells (Morohashi *et al* 1995, Heath *et al* 1997, Ochensberger *et al* 1999, Nilsson *et al* 1999, Sozzani *et al* 1997). Although there are 2 forms of human IL-8, there has yet to be identified a mouse or rat homologue. The existence of such a rodent counterpart is suggested by inhibition of lung inflammation in rats with neutralising antibodies to human IL-8

(Mulligan *et al* 1993). Cacalano identified a reputed mouse gene with close homology to CXCR1 and CXCR2, and on the examination of IL-8R null mice they observed an expansion in neutrophilic myeloid cells, B cells and enhanced myelopoiesis in the bone marrow (Cacalano *et al* 1994). This lead to the suggestion that the signalling through the murine IL-8 receptor was involved in the negative regulation of B cells and neutrophil production and this is consistent with the negative regulatory effects that human IL-8 has on myeloid cells *in-vitro* (Lu *et al* 1993). A further study by Broxmeyer *et al* also indicated that murine IL-8 receptor knockout myeloid progenitors were insensitive to human IL-8 or MIP-2 providing further evidence for the negative regulatory role of mIL-8R (Broxmeyer *et al* 1996).

2.4.2 Stromal Derived Factor 1 (SDF-1)

Stromal derived factor (SDF-1) or pre-B cell growth stimulatory factor (PBSF) was originally cloned from bone marrow stromal cell lines using a signal sequence trap technique (Tashiro *et al* 1993). Alternative splicing produces two isoforms of SDF-1, SDF-1α and SDF-1β, which differ by four C-terminal amino acids (Shirozu *et al* 1995). Similarly to Mig and IP-10, SDF-1α and β do not contain the ELR motif prior to the first cysteine residue and thus do not induce neutrophil migration (Liao *et al* 1995). SDF-1α and β have been reported to chemoattract lymphocytes, macrophages and as the original name pre-B cell growth stimulatory factor suggests, they can stimulate B cell progenitor proliferation *in-vitro* (Bleul *et al* 1996). SDF-1 has proved to be unique among the chemokines, in that it is constitutively expressed by many tissues and it is highly conserved between species e.g. human and mouse SDF-1 are 99% identical in sequence (Shirozu *et al* 1995).

In-vitro studies observed that SDF-1 is a chemotaxin for $CD34^+$ haemopoietic cells and cells of the megakaryocyte lineage, isolated from the bone marrow and peripheral blood (Wang *et al* 1998). Although SDF-1 has been observed to induce migration of haemopoietic progenitor cells, SDF-1 alone or in combination with IL-3 and SCF displays no enhancement of colony formation. However, SDF-1 has been observed to decrease the colony formation of a myeloid progenitor cell line, 32D cells (Sanchez *et al* 1997) suggesting that it may play a role in regulation of myeloid progenitors *in-vivo*. Further work has also indicated that SDF-1 can induce adhesion of

megakaryocytes to bone marrow endothelial cells and enhance megakaryocyte progenitor migration (Wang *et al* 1998). Furthermore, Campbell *et al* also demonstrated that SDF-1 could induce adhesion of lymphocytes to endothelial cells (Campbell *et al* 1998). These various studies indicate that SDF-1 may be involved in trafficking of mature blood cells and, migration or homing of $CD34^+$ progenitors to different niches during the differentiation and maturation of haemopoietic progenitor cells. A further activity of SDF-1 is its ability to inhibit HIV-1 from infecting their target cells (Bleul *et al* 1996) this will be discussed later in section 2.10. In an attempt to further examine the role of SDF-1, inactivation of the PBSF/SDF-1 gene and examination of the resultant embryos was reported by Nagasawa *et al* (Nagasawa *et al* 1996). These SDF-1 null mice died perinatally and were observed to have a reduced level of B cell progenitors in both the liver and bone marrow and a reduction in myelopoietic progenitors in the bone marrow. These SDF-1 null mice also displayed major defects of the heart, the large blood vessel supplying the gastrointestinal tract, and the brain. Upon further examination of earlier developing embryos from these SDF-1 null mice, it was observed that these embryos had normal fetal liver myelopoiesis, suggesting that the decrease in bone marrow myelopoiesis is possibly due to the impaired migration of myeloid stem/ progenitor cells from the fetal liver to the bone marrow (Nagasawa *et al* 1996).

These multiple functions of SDF-1 are induced through its interaction with its receptor CXCR-4. CXCR4 was previously known as LESTR and Fusin (Loetscher *et al* 1994, Feng *et al* 1996) however, subsequent work identified SDF-1 as the ligand for both of these receptors and therefore, this receptor was formally renamed CXCR4 (Bleul *et al* 1996, Oberlin *et al* 1996). CXCR4 is widely expressed in the various mature cell types of the haemopoietic system e.g. neutrophils, monocytes, T and B cells and expression has also been observed in less mature cell types such as B cell precursors and $CD34^+$ progenitor cells (Zaitseva *et al* 1997, 1998). Outwith the haemopoietic system, CXCR4 has been observed to be expressed on vascular endothelial cells, in both microglia and astrocytes in the brain and neurones in the peripheral and central nervous system (Gupta *et al* 1998, He *et al* 1997). Interestingly CXCR4 null mice display a similar phenotype to SDF-1 null mice, suggesting that the interaction of SDF-1 and CXCR4 is unique and non promiscuous *in-vivo*, and that their interaction is involved in homing of stem cells to their niches.

Figure 2.1 An up-to-date list of the Human and Murine Chemokines

CC Chemokines

Human	Mouse
I-309	TCA-3
MCP-1	JE
MIP-1α	MIP-1α
MIP-1β	MIP-1β
RANTES	RANTES
Unknown	C10/MRP-1
MCP-3	MARC?
MCP-2	MCP-2?
Unknown	MRP-2/MIP-1δ
Eotaxin	Eotaxin
Unknown	MCP-5
MCP-4	Unknown
HCC-1	Unknown
HCC-2	Unknown
HCC-4	Unknown
TARC	TARC
PARC/DC-CK1	Unknown
ELC/MIP-3β	ELC/MIP-3β
LARC/MIP-3α	LARC/MIP-3α
SLC	SLC
MDC	ABCD-1
MPIF-1	Unknown
Eotaxin-2	Unknown
TECK	TECK
Eotaxin-3	Unknown
CTACK/ILC/	CTACK/ILC/Eskine

CXC Chemokines

Human	Mouse
GRO-α	GRO/KC?
GRO-β	GRO/KC?
GRO-δ	GRO/KC?
PF-4	PF-4
ENA-78	LIX?
GCP-2	CKα-3
NAP-2	Unknown
IL-8	Unknown
Mig	Mig
IP-10	IP-10
I-TAC	Unknown
SDF-1 α/β	SDF-1
BCA-1	BCA-1
BRAK	BRAK
Unknown	Lungkine

CX_3C Chemokines

Human	Mouse
Fractalkine	Neurotactin

C Chemokines

Human	Mouse
Lymphotactin/SCM-1α	Lymphotactin
SCM-1β	

Figure 2.1 An up-to-date list of the Human and Murine Chemokines

? Possible murine equivalent, if one exists

2.5 The C-C Chemokines

The C-C chemokine superfamily is by far the largest chemokine subfamily containing somewhere in the region of 28 members. The first C-C chemokine was isolated by differential hybridisation from human tonsillar lymphocytes and was known as LD78 (Obaru *et al* 1986). Several cDNA isoforms of a closely related protein Act-2 were later identified (Lipes *et al* 1988). Due to the amino acid identity of more than 70%, the murine proteins MIP-1α and MIP-1β were considered as the homologues of LD78 and Act-2 (Sherry *et al* 1988), and recently the names MIP-1α and β have been used to describe both the murine and human forms of the proteins. Like the α chemokines (CXC), the β chemokines (CC) can be further divided into inducible pro-inflammatory chemokines such as MIP-1α and RANTES which can be produced in response to microbial, inflammatory or immune signals, and account for the increased leukocyte recruitment under these conditions, and constitutive homeostatic chemokines such as HCC-1, SLC and ELC, which are hypothesised to control the normal trafficking of leukocytes under physiological conditions. Other examples of CC chemokines are RANTES (Regulated on activation normal T expressed and secreted) (Schall *et al* 1988), I-309 (Miller *et al* 1989), HCC-1 (Schulz-Knappe *et al* 1996) and MCP-1 (Yoshimura *et al* 1989 a,b,). Due to the use of techniques referred to earlier the expansion in numbers of CC chemokines has been more dramatic in recent years; TARC (Imai *et al* 1996), TECK (Vicari *et al* 1997), Eotaxin-2 (Forssmann *et al* 1997), MCP-4 (Uguccioni *et al* 1996), secondary lymphoid chemokine (SLC) (Nagira *et al* 1997), Epstein-barr virus 1 chemokine (ELC) (Yoshida *et al* 1996), MPIF (Patel *et al* 1997), ESkine (Baird *et al* 1999) and for further examples of CC chemokines the reader is referred to a review by Zlotnik (Zlotnik *et al* 1999). Historically, the C-C chemokines were described as monocyte and macrophage chemotaxins, however, it is now accepted that C-C chemokines can induce chemotaxis of a variety of leukocyte populations. As was the case for the CXC chemokines, the discussion of only two CC chemokines relevant to this project is presented in more detail below.

2.5.1 RANTES (Regulated upon Activation of Normal T cell Expressed and Secreted)

RANTES was isolated by Schall *et al* from a differential screen using T cells versus B cells (Schall *et al* 1988). RANTES is a basic protein composed of 69 amino acids which contains a 23 amino acid signal peptide and upon cleavage a mature protein of approximately 7.8 kDa is generated. The murine RANTES gene is localised on chromosome 11 (Danoff *et al* 1994), whereas, the human gene resides on chromosome 17 (Nelson *et al* 1993). Murine and human RANTES share 85 % amino acid identity and this may explain the observed species cross reactivity of RANTES (Schall *et al* 1992).

RANTES is produced by a variety of cells such as T cells, natural killer (NK) cells, platelets (Klinger *et al* 1995), eosinophils (Lim *et al* 1996) and epithelial cells (Schall 1991). RANTES expression has also been detected from synovial fibroblasts isolated from patients with Rheumatoid arthritis (RA) (Rathanswami *et al* 1993), and the expression of RANTES in these cells can be further stimulated by TNF-α and IFN-γ.

RANTES was initially identified as a chemotaxin for monocytes and memory T cells but not neutrophils (Schall *et al* 1990), however, further studies indicated that RANTES can chemoattract a wide variety of leukocyte subsets e.g., eosinophils (Rot *et al* 1992), platelets (Kameyoshi *et al* 1992), basophils (Bischoff *et al* 1993), NK cells (Taub *et al* 1995), $CD4^+$ and $CD8^+$ T cells (Roth *et al* 1995) and memory T cells $CD4^+$ CD45 RO^+ (Schall *et al* 1990). RANTES may also play an important role in trans endothelial migration of leukocytes as RANTES has been observed to induce adhesion of monocytes and T cells (Vaddi *et al* 1994) to recombinant ICAM-1 and VCAM-1 (Lloyd *et al* 1996), and can regulate the secretion and activity of matrix metalloproteinase 2 and 9 (Xia *et al* 1996). RANTES induces its functions through the chemokine receptors CCR1, 3, 5 and D6 and these are discussed in section 2.6.1.

RANTES has been implicated in a number of auto-inflammatory diseases, e.g. experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), which is characterised by auto-reactive T cells infiltrating the CNS (Miyagishi *et al* 1997). Following antigen recognition, these cells become activated

and secrete their pro-inflammatory cytokines TNF-α and IL-1 which in turn lead to the induction of chemokines such as MIP-1α and RANTES and the subsequent recruitment of additional inflammatory cells into CNS (Ranschoff *et al* 1996). RANTES has also been implicated in other inflammatory diseases e.g. Rheumatoid Arthritis (Kunkel *et al* 1996), asthma which leads to Allergic Airway inflammation (AAI) (Luckas *et al* 1996a), and skin inflammation (Schroder *et al* 1996). Further evidence for RANTES involvement in these inflammatory states was observed by the ability of a RANTES derivative Met-RANTES to reduce the onset of collagen induced RA, and also reduce the leukocyte infiltration in AAI (Plater-Zyberk *et al* 1997, Gonzalo *et al* 1998). These observations indicate that RANTES may be pathogenically responsible for the manifestations of these diseases.

2.5.2 Macrophage Inflammatory Protein 1 Alpha (MIP-1α)

MIP-1α was initially identified as a part of a complex known as macrophage inflammatory protein (MIP) which was released from a murine macrophage cell line after LPS stimulation. (Wolpe *et al* 1988). Subsequent fractionation proved MIP activity contained two closely related proteins, MIP-1α and MIP-1β, and their cDNA clones were isolated by Sherry *et al* (Sherry *et al* 1988). Human MIP-1α, previously known as LD78, is 92 amino acids in length and contains a 22 amino acid signal peptide (Obaru *et al* 1986). Upon cleavage, the secreted product is a 70 amino acid polypeptide, which has a molecular weight of about 7.8 kDa. Murine MIP-1α is a single copy gene and is located on chromosome 11 clustered with the majority of the other members of the C-C chemokine family. In humans, unlike the mouse, there are three distinct isoforms of MIP-1α (LD78 α, β and γ). They are clustered with the majority of other β chemokines on chromosome 17 in the region q11-q21. The α gene exists, as a single copy however, there can be anywhere between 0 and 6 copies of the β gene on chromosome 17 (Irving *et al* 1990). The product of the LD78α gene was initially thought to be the human homologue of mMIP-1α. However, a recent study by Nibbs *et al* indicated that upon analysis of receptor binding, the product of the LD78β gene was more closely related to mMIP-1α than LD78α (Nibbs *et al* 1999). Nibbs *et al* also observed that LD78β is a more efficient inhibitor of HIV infection compared to LD78α, and this was further confirmed by a study by Menten *et al* which indicated that LD78β was more potent at inhibiting HIV infection than LD78α, MIP-

1β or RANTES (Menten *et al* 1999). These studies therefore, suggested that LD78β is the human homologue of murine MIP-1α and not LD78α as initially proposed. Furthermore, the observation that certain individuals have several copies or no copies of the LD78β gene suggests that these individuals may be less or more susceptible to becoming HIV positive. Unlike LD78 α and β, LD78 γ cannot produce mRNA and is therefore an unexpressed pseudogene. It also differs from the α and β forms by having its upstream control region and most of its first intron missing, this may explain why LD78γ cannot be expressed. Similarly to LD78β, LD78 γ can be present in a various copies on chromosome 17q11-q21 and interestingly in some individuals it is missing (Irving *et al* 1990).

Initial observations indicated that MIP-1α and MIP-1β like other chemokines such as RANTES have the propensity to form high molecular weight aggregates in physiological buffers, and that these molecules could exist as multimers with molecular mass in excess of 100 kDa. This self aggregation is dependant on the protein concentration and the buffer in which the peptide is dissolved and is a consequence of non covalent, and electrostatic interactions as reversal of the

aggregation can be observed at high salt concentrations (Patel *et al* 1993, Graham *et al* 1993). Graham *et al* produced monomeric, dimeric and tetrameric forms of mMIP-1α by sequentially neutralising carboxyl terminal acidic amino acid residues and observed that these variants, when compared to the wild type protein, have similar activities in CFU-A colony forming assays and monocyte shape change assays (Graham *et al* 1994). They therefore suggested that the active form of MIP-1α must be the monomeric form and that the high molecular weight aggregates, dissaggregate in dilute solution. A similar study using human MIP-1α indicated that disaggregation did not effect the activity of the molecule. Indeed, Czaplewski and colleagues produced a MIP-1α mutant, by a single amino acid substitution Asp26 to Ala26, which they referred to as BB10010 and had an average native molecular weight of 19 kDa (Czaplewski *et al* 1999).

2.5.2.1 MIP-1α Expression

In un-stimulated cells it is difficult to detect MIP-1α mRNA and protein, however, with the use of sensitive PCR, MIP-1α mRNA has been reported to be detectable at low levels within a wide variety of haemopoietic and non haemopoietic cell types and in the blood and bone marrow of healthy individuals (Cluitsman *et al* 1995). This suggests that there is a basal level of MIP-1α in normal tissue in the absence of any overt inflammation and the probable source is monocytes and tissue macrophages.

A number of different stimuli such as LPS, IL-3, IFN-γ, M-CSF, GM-CSF, adhesion molecules and antibodies can cause rapid and transient induction of MIP-1α transcription and translation in numerous cell types which include macrophages (Maltman *et al* 1993, 1996, Jarmin *et al* 1999), neutrophils (Alam *et al* 1992), basophils (Li *et al* 1996), mast cells (Yano *et al* 1997), B cells (Sharma *et al* 1997), T cells (Maric *et al* 1997) and platelets (Klinger *et al* 1995). A more recent study by Majka *et al* also demonstrated that human $CD34^+$ cells and $CD34^+$/c-kit^+ cells that are enriched for myeloid precursors can express and secrete MIP-1α, MIP-1β and RANTES, and upon stimulation with IFN-γ the level can be increased (Majka *et al* 1999). Other studies have also detected MIP-1α in non-haemopoietic cell types such as primary human fibroblasts (Nakao *et al* 1990), epidermal langerhan cells (Parkinson *et al* 1993), glioma cells and microglial cells (Hayashi *et al* 1995, Tanabe *et al* 1997). As well as inducing MIP-1α expression, there are also a number of factors that have been observed to inhibit MIP-1α production, indeed IL-10, IL-4 and IL-13 inhibit MIP-1α production by inducing mRNA degradation in peripheral mononuclear cells, human blood monocytes and alveolar macrophages (Berkman *et al* 1995, 1996, Standiford *et al* 1993). A study by Maltman *et al* reported that TGF-β down regulated the expression of MIP-1α (Maltman *et al* 1993), and on further analysis Maltman defined an endogenous reciprocal relationship between MIP-1α and TGF-β in bone marrow macrophages whereby, elevation of MIP-1α lead to the increase in TGF-β which in turn lead to the suppression of MIP-1α. Thus, in the bone marrow where TGF-β levels are easily detected, TGF-β may act as a control element and keeps the level of MIP-1α in check (Maltman *et al* 1996). Furthermore, Jarmin *et al* observed that IL-3 and GM-CSF can induce the expression of MIP-1α in bone marrow

macrophages and that the level of MIP-1α expression is reduced in the presence of TGF-β (Jarmin *et al* 1999). Interestingly, TGF-β has been observed to down regulate the expression of GM-CSF and IL-3 receptors as well as CCR1, a MIP-1α receptor. Therefore, TGF-β can interfere with MIP-1α activity by reducing its expression, by down regulating the expression of its receptors and by down regulating the receptor expression of growth factors that up regulate MIP-1α expression. In contrast to the above model of MIP-1α regulation, McManus *et al* observed that expression of MIP-1α and MIP-1β can be induced by LPS in human fetal microglia and that TGF-β could not modulate their expression (McManus *et al* 1998). Therefore the relationship between MIP-1α and TGF-β demonstrated *in-vitro* might be more complicated *in-vivo*, where MIP-1α expressing cells will ultimately be exposed to a wide range of interacting growth factors.

2.5.2.2 Inflammatory Effects of MIP-1α

Historically, CC chemokines have not been observed to chemoattract neutrophils, however, the injection of high concentrations of MIP-1 into the footpads of mice was observed to induce neutrophil infiltration into the injection site (Wolpe *et al* 1988). Additionally, McColl *et al* indicated that human MIP-1α had limited ability as a neutrophil chemoattractant (McColl *et al* 1993) and more recently murine MIP-1α was observed to induce both chemotaxis and calcium release in murine neutrophils (Gao *et al* 1997). Furthermore, Zhang *et al* observed that human MIP-1α can only induce a small release of calcium, and cannot induce chemotaxis in human neutrophils (Zhang *et al* 1999). The confusion in neutrophil chemoattraction by MIP-1α may be explained if MIP-1α does not play a role in neutrophil chemoattraction in humans but does in mice. IL-8 in humans may have evolved to play a more specific role in human neutrophil chemoattraction than MIP-1α, and as mice do not have a homologue of IL-8, MIP-1α may fulfil the role of IL-8 in mice. These studies indicate that one has to be cautious in assuming that the functions of a chemokine in mouse are the same as in man.

MIP-1α in common with other chemokines has multiple activities on a range of leukocytes. MIP-1α has been observed to chemotax monocytes and $CD8^+$ T lymphocytes (Fahey *et al* 1992, Taub *et al* 1993, Schall *et al* 1993), as well as being a

chemotaxin for dendritic cells, NK cells, basophils and mast cells, it also activates these cells (Alam *et al* 1994, Luckas *et al* 1995a) and MIP-1α can enhance the proliferation and activation of T lymphocytes (Taub *et al* 1996). Furthermore, MIP-1α can also induce the expression of cytokines such as TNF-α, IL-1α and IL-6 from macrophages (Fahey *et al* 1992). Antigen activation of the B cell receptor triggers the expression of MIP-1α and MIP-1β and as B cell maturation is ultimately dependant on T cell help, it is possible that the expression of MIP-1α and β two T cell chemoattractant agents (Tedla *et al* 1998) may be one mechanism that helps bring these two cell types together (Krzysiek *et al* 1999). These observations indicate that MIP-1α has multiple activities on a range of leukocytes and that MIP-1α acts at the onset of inflammation and can be clearly seen as an important immuno-regulatory cytokine.

In order to examine the biological role of MIP-1α *in-vivo*, Cook *et al* (Cook *et al* 1995) generated MIP-1α null mice. Mice homozygous for this disruption revealed no obvious abnormalities in development or haemopoiesis, thus suggesting that MIP-1α is not required to maintain normal stem cell quiescence and other stem cell inhibitors must compensate, since the cycling rates of the stem cells from MIP-1α null mice is comparable to that seen in wild type animals. However, these MIP-1α null mice were shown to have a phenotype but only after infection with influenza or coxsackie viruses. MIP-1α was observed to be essential for the effective control of the influenza infection, as MIP-1α null mice infected with influenza were observed to have a reduced inflammatory response and a delayed clearance of the virus from their lungs (Cook *et al* 1995). Furthermore, the characteristic myocarditis observed in wild type mice infected by coxsackie B virus did not occur in MIP-1α null mice. The observation that T cells are needed for the viral clearance from lungs and the possible involvement of cytotoxic T cells in the cardiac lesions associated with myocarditis indicates that MIP-1α maybe the essential molecule needed for recruitment of these cells. A further study by Cook *et al* also observed the role of T cells and MIP-1α in the clearance of the intracellular pathogen *Listeria monocytogenes in-vivo* (Cook *et al* 1999).

2.52.3 MIP-1α as a Haemopoietic Regulator

MIP-1α has a number of other activities outwith the pro-inflammatory actions on the mature blood cell system. MIP-1α can reversibly inhibit stem cell proliferation, however, its inhibitory properties are restricted to the transiently engrafting stem cells. Within the stem cell compartment, a range of *in-vitro* assays have demonstrated MIP-1α's ability to inhibit the growth of cells detected in CFU-GEMM, BFU-E and CFU-S/A assays and its inability to inhibit proliferation of more primitive cell types such as long term repopulating stem cells and HPP-CFC 1 (Su *et al* 1997, Graham *et al* 1990, Quesniaux *et al* 1993, Keller *et al* 1994). Removing lineage positive cells from bone marrow and enriching the remaining lineage negative cells on the basis of expressing an antigen known as Thy-1, indicated that MIP-1α could not inhibit the mature Thy-1^{+} Lin $^{-}$ cells, however, MIP-1α could inhibit the Thy-$1^{lo/-}$ Lin^{-} population (Keller *et al* 1994). Su *et al* observed that MIP-1α can inhibit the formation of BFU-E but not CFU-E from human bone marrow and suggested that this may be through the interaction with a known MIP-1α receptor CCR1 as the observed inhibition could be reversed by anti CCR1 antibodies (Su *et al* 1997). However, Broxmeyer *et al* indicated that CCR-1 is not the dominant receptor for MIP-1α suppression of myeloid progenitors such as CFU-GEMM, CFU-GM or BFU-E in mice (Broxmeyer *et al* 1999). These contradictory results suggest that the interaction between MIP-1α and CCR1 and the functional outcome may be different in the human and murine systems.

In-vivo studies have also indicated that MIP-1α can function as an inhibitor of stem cell proliferation. Indeed, Dunlop *et al* observed that MIP-1α could protect the CFU-S stem cells following treatment with the cytotoxic drug cytosine arabinoside (ARA-C) and furthermore, MIP-1α was observed to inhibit the proliferation of d12 CFU-S stem cells (Dunlop *et al* 1992). MIP-1α suppression was further substantiated *in-vivo* in mice (Lord 1992) and was confirmed in breast cancer patients by an analogue of MIP-1α with improved solubility properties, BB10010 (Broxmeyer *et al* 1998). However, data from other clinical trials have suggested that MIP-1α, BB-10010, displays no myelosuppressive activities (Bernstein *et al* 1997, Clemons *et al* 1998). A further activity of MIP-1α was observed upon systemic injections of BB-10010, which produced a mobilisation of progenitor cells to the peripheral blood in mice and

humans (Lord *et al* 1995, Broxmeyer *et al* 1998). As well as the possibility of the myeloprotective activity of MIP-1α being useful as a therapeutic in cancer treatment, the increased mobilisation of stem cells to the peripheral blood may also prove to be useful in leukopheretic protocols prior to ablative therapies. An additional therapeutic role for MIP-1α lies in the treatment of some leukaemia's. Eaves and colleagues have indicated that MIP-1α does not inhibit the proliferation of primitive CML cells, but can inhibit the proliferation of normal primitive progenitors supported in long-term cultures (Eaves *et al* 1993, Chasty *et al* 1995). Chronic myeloid leukaemia (CML) is a clonal disorder of the pluripotent haemopoietic stem cell, which is characterised by the constitutively activated Bcr-Abl protein tyrosine kinase (PTK). A further study by Wark *et al* using FDCP-Mix cells transfected with a temperature sensitive v-abl PTK, indicated that MIP-1α could inhibit these cells at the restrictive temperature and could protect these cells from death due to exposure of cytotoxic agents. However, at the permissive temperature for abl PTK activity, MIP-1α treatment did not inhibit the proliferation of these cells and could not protect these cells from cell death induced by cytotoxic drugs (Wark *et al* 1998), thus providing further evidence for the possible treatment of CML with a regime of cytotoxic agents and MIP-1α.

As well as growth inhibitory properties MIP-1α has been observed to exert growth-promoting effects on some cells of the haemopoietic system. These growth-stimulating functions depend on the maturation state of the cellular population and the presence of specific growth conditions. Indeed, DeWynter *et al* isolated $CD34^+$ cells from human bone marrow and cord blood and observed that MIP-1α could inhibit CFU-GM from $CD34^+$ human bone marrow and stimulate the growth of CFU-GM from cord blood (DeWynter *et al* 1998). Furthermore, Broxmeyer *et al* also reported that *in-vitro* MIP-1α could enhance CFU-GM and CFU-M progenitor colony formation (Broxmeyer *et al* 1989, 1990 and 1999). In non stromal contact long term bone marrow cultures, MIP-1α in the presence of IL-3 and diffusable soluble stromal factors has also been observed to maintain the numbers of long term repopulating cells, and it has been suggested that this may be due to the inhibition of differentiation (Verfaillie *et al* 1995). In addition to the inhibition of CFU-A/S stem cells, MIP-1α has been observed to function as a potent and reversible inhibitor of human keratinocyte proliferation (Parkinson *et al* 1993). Epidermal langerhan cells and not keratinocytes are the source of MIP-1α, and it is hypothesised that MIP-1α regulates

the proliferation of the stem cells within the skin (Graham *et al* 1992). High levels of MIP-1α have been detected in and around wounds suggesting a possible role for MIP-1α in wound healing (Dipetro *et al* 1998). These studies indicate that as well as MIP-1α inhibiting cell proliferation of transiently engrafting stem cell it can also act as a growth stimulator.

2.5.2.4 MIP-1α and Diseases

The wide range of biological functions of MIP-1α indicates that its uncontrolled expression may play a role in inflammatory diseases. Indeed, MIP-1α neutralising antibody studies have been used to demonstrate that MIP-1α is involved in a number of autoimmune diseases. MIP-1α antibodies can reduce the neutrophil infiltration in rats with LPS induced acute lung injury and also reduce the granuloma formation around the ova of the Schistosome parasite (Shanley *et al* 1995, Lukacs *et al* 1993). Furthermore, the administration of MIP-1α antibody attenuated the influx of eosinophils in a mouse model of airway inflammation, suggesting an *in-vivo* role for MIP-1α in eosinophillic inflammation (Rot *et al* 1992, Lukacs *et al* 1995).

Allergic inflammation is characterised by the presence of activated eosinophils, basophils and T cells and since MIP-1α attracts and activates eosinophils, basophils, lymphocytes and monocytes it may play a role in the pathogenesis of allergic inflammation. Inflammatory lung disease can be induced experimentally in lab animals and it is associated with considerable up regulation of MIP-1α expression. The exacerbation of asthma into allergic airway inflammation (AAI) is dependant on antigen challenge and the generation of inflammatory mediators. These inflammatory mediators and chemotactic factors can activate various populations of leukocytes and the recruitment and activation of eosinophils is thought to be responsible for the adverse effects in AAI. It has been shown that the CC chemokines MIP-1α, RANTES, Eotaxin and MCP-3 are potent eosinophil chemotaxins *in vitro* (Jose *et al* 1994, Rothenburg *et al* 1997, Dahinden *et al* 1994). However, the specific contribution of these chemokines in the pathology of AAI is not known. Luckas and Kunkel developed a murine model of AAI and they observed that antibodies against MIP-1α and RANTES (Luckas *et al* 1995b) reduced eosinophil recruitment during the pathogenesis of the disease.

MIP-1α is also involved in Rheumatoid arthritis (RA). This autoimmune disease is characterised by a recruitment and activation of leukocyte subsets in the synovial fluid of joints. In patients suffering from RA, it has been shown that the synovial chondrocytes are an abundant source of MIP-1α (Pulsatelli *et al* 1999, Borzi *et al* 1999). In combination with other chemokines, MIP-1α is known to play a role in the infiltration of leukocyte subsets into tissue and is thought to be involved in the pathogenesis of RA. An animal model of RA, Murine type 2 collagen induced arthritis, also shows an up regulation of MIP-1α expression and this increases as the incidence and severity of the disease increases, and significantly the extent of the disease is reduced when the mice are treated with monoclonal antibodies to MIP-1α (Thornton *et al* 1999)

There is evidence both *in-vitro* and *in-vivo* for the production of MIP-1α in the CNS, Hayashi *et al* indicated that MIP-1α expression was induced in microglial cells after stimulation with LPS and Miyagishi observed that MIP-1α was present in the cerebrospinal fluid of MS patients (Hayashi *et al* 1995, Miyagishi *et al* 1995). MIP-1α can also induce calcium release and chemo-attraction in astrocytes (Tanabe *et al* 1997). Further evidence for the expression of MIP-1α in brain was observed in HIV encephalitis and schizophrenia patients (Shmidtmayerova *et al* 1996, Ishizuka *et al* 1997). Indeed, Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the CNS, which serves as a model for the human disease multiple sclerosis (MS). In both diseases circulating leukocytes penetrate the blood brain barrier, and the subsequent demyelination of neurones which is mediated by T cells results in impaired nerve conduction and paralysis (Macfarlin *et al* 1983, Karin *et al* 1993). In this condition it is the recruitment of inflammatory cells by MIP-1α and MCP-1 that contributes to the pathogenesis of the disease. Further evidence for the role of MIP-1α in EAE was demonstrated by Karpus *et al*, they indicated that antibodies to MIP-1α could prevent the development of EAE (Karpus *et al* 1995).

2.6 Chemokine Receptors and Binding Proteins

Chemokine receptors belong to the seven transmembrane spanning family of G-protein coupled receptors. To date there are 20 human chemokine receptors, many of which exhibit multiple ligand specificity, indicating the redundancy and versatility of

the chemokine system. Five human CXC chemokine receptors (CXCR1 through CXCR5), 11 human CC chemokine receptors (CCR1-11), one human CX_3C and one human XCR1 have been identified thus far (Zlotnik *et al* 1999). An atypical human and murine receptor (D6) has been cloned by Nibbs *et al.* This receptor displays the greatest promiscuity of all the chemokine receptors, and thus far it has not been shown to signal in response to ligand binding (Nibbs *et al* 1997a, b). In addition to these receptors, there is a chemokine binding protein expressed on the surface of red blood cells and post capillary high endothelial cells known as Duffy antigen receptor for chemokine (DARC). This is a 7 transmembrane receptor that does not appear to signal upon interaction with various chemokines and thus may be coupled to different signalling transducing pathways. Although the chemokine/ ligand promiscuity does not usually cross the CC and CXC boundaries one exception to the rule is the Duffy Antigen Receptor for Chemokines (DARC) (Hadley and Peiper 1997).

Common features of chemokine receptors include; a conserved primary amino acid homology 36-77 % in CXC and 46-89 % with CC receptors, an acidic N terminus and a DRYLAIVHA sequence or a variation of it in the second intracellular loop. Furthermore, these receptors also have two conserved cysteines (Cys) residues in their primary amino acid sequence, one in the NH_2 terminal domain and one in the third extracellular loop, and these cysteine residues form a disulphide bond which is critical for the conformation of the ligand pocket. The major shared biological function of the chemokine receptors is leukocyte trafficking and dependent processes such as immune surveillance, innate and adaptive immune responses (Foxman *et al* 1997). Within these areas each chemokine receptor appears to have a specific role, which is determined by their expression pattern on specific leukocytes and the temporal and spatial specificity of its ligand expression. The known chemokine receptors all appear to exhibit overlapping specificity, figure 2.2, albeit with variable affinities for their different ligands. As indicated above there is a number of chemokine receptors however, within the context of this project the relevant receptors are the receptors that interact with MIP-1α and RANTES and these are described in more detail in section 2.6.1. For further information on chemokine receptors the reader is referred to a more recent review on the chemokine receptors by Murphy *et al* (Murphy *et al* 2000).

Interestingly chemokine receptors are not the only proteins that chemokines can interact with. Chemokines are highly basic proteins and because of this a further

property of most chemokines is their ability to interact with heparin and glycosaminoglycans (Koopman and Krangel 1997, Koopman *et al* 1999). Indeed, numerous studies have indicated that various chemokines such as IL-8, PF-4, MIP-1α, MIP-1β and RANTES can interact with heparin (Mayo *et al* 1995, Koopman and Krangel 1997, Koopman *et al* 1999). The immobilisation of chemokines by the cell surface proteoglycans or components of the extracellular matrix is thought to be important for the maintenance of the immobilised or haptotactic gradient, which is needed for leukocyte activation, diapedesis and migration into the tissue (Rot *et al* 1993). However, a further role for GAG binding was suggested by Kuschert *et al*, they observed that soluble GAGs could form complexes with chemokines and that these complexes could inhibit chemokine receptor binding and cellular responses of these chemokines (Kuschert *et al* 1999). Until recently, no data has been obtained to directly indicate a role for these interactions *in-vivo* however, a study by Middleton *et al* indicated that heparan sulphate can interact with IL-8 *in-vivo* (Middleton *et al* 1997).

In an attempt to analyse the function of the heparin-binding site in MIP-1α, Graham *et al* produced a variant of mMIP-1α that had its stem cell inhibitory activity uncoupled from its ability to chemoattract monocytes. Graham and colleagues named this molecule HepMut, this molecule has a mutation of two basic amino acids in its proteoglycan binding domain which renders this molecule unable to bind to a range of proteoglycans. HepMut was observed to be as good as wild type MIP-1α at inhibiting CFU-S / A cell proliferation, however, it was unable to induce monocyte shape change or bind to the MIP-1α receptor CCR1 (Graham *et al* 1996), thus suggesting that the binding of proteoglycans is not essential for MIP-1α induced stem cell inhibitory activity but may be involved in monocyte chemoattraction through its interaction with CCR1. A similar study by Koopman *et al* identified a single basic residue that is involved in the binding of human MIP-1α to glycosaminoglycans (GAGs). They observed that the ability to bind to GAGs was not essential for receptor binding or signalling *in-vitro* by MIP-1α and unlike HepMut this mutant could bind to CCR1. This may be due to species differences or due to the different nature of the mutagenesis (Koopman and Krangel 1997). This also suggests that GAG binding is not necessary for the activity of MIP-1α, however, all chemokines can bind to heparin

Figure 2.2 The Human Chemokine Receptor Family and Associated Ligands

Receptor	Ligands
CXCR1	IL-8, GRO-α, GCP-2
CXCR2	IL-8, GRO-α, β and δ, NAP-2, ENA-78, GCP-2
CXCR3	IP-10, MIG, I-TAC
CXCR4	SDF-1α and β
CXCR5	BCA-1
CCR1	MIP-1α, β, RANTES, MCP-3, HCC-1, 2 and 4, MPIF-1
CCR2	MCP-1, 3, 4
CCR3	Eotaxin, Eotaxin 2, 3, RANTES, MCP-2, 3, 4, HCC-2
CCR4	TARC, MDC
CCR5	MIP-1α, MIP-1β, RANTES, MCP-2
CCR6	MIP-3α
CCR7	ELC, SLC
CCR8	I-309
CCR9	TECK
CCR10	CTACK
CCR11	SLC, TECK
D6	MIP-1α, β, RANTES, MCP-1, 2,3,4, Eotaxin, HCC-1
XCR1	Lymphotactin, SCM-1β
CX3C1	Fractalkine
Duffy	RANTES, MCP-1, IL-8

and it is possibly involved in the presentation of chemokines in sites of active inflammation (Hoogeworf *et al* 1997). A further study by Oravecz *et al* suggested that GAGs were important in the inhibitory activity of RANTES and MIP-1α in HIV infection, as removal of these GAGs resulted in the inability of RANTES and MIP-1α to inhibit HIV infection (Oravecz *et al* 1997). This result suggested that either the presentation of these chemokines to CCR5 or indeed, the increased local concentration produced by the interaction of the chemokine with the GAG is important in the mechanism of HIV inhibition. These studies indicate that chemokine binding to GAGs is not always necessary for their function, however, they do suggest that these molecules may have a role to play in some aspects of chemokine function.

2.6.1 The C-C Receptors used by MIP-1α and RANTES

2.6.1.1 CCR1

CCR1 was originally designated MIP-1α/RANTES receptor based on its high affinity for these ligands. The CCR1 gene is on human chromosome 3 clustered with several other chemokine receptors such as CCR3, CCR5, and several orphan receptors (Samson *et al* 1996). Human CCR1 can bind multiple inflammatory/inducible human CC chemokines with similar high affinities, MIP-1α, RANTES, MCP-2 and 3, leukotactin-1/ MIP-5 and MCIF-1, and also bind other ligands such as MIP-1β, MCP-1 and HCC-1 with lower affinities, as indicated in figure 2.2. Mouse CCR1 displays an 80 % sequence identity to the human receptor and has been observed to bind both human and murine MIP-1α with high affinity (Post *et al* 1995, Zhang *et al* 1999). CCR1 is expressed on monocytes, macrophages and T-lymphocytes, furthermore there is an increased expression of CCR-1 on $CD45RO^+$ memory T cells compared to that on CD45 RO^- T cells (Sallusto *et al* 1999). Pre-treatment of monocytes with IL-10 leads to an increased expression of CCR1, as well as CCR3 and CCR5, and this is due to IL-10 prolonging the half-life of the mRNA (Sozzani *et al* 1998, Zella *et al* 1998). Furthermore, Graham *et al* have observed that both human and murine MIP-1α do not induce the inhibition of proliferation of CFU-A /S stem cells through CCR1 (Graham *et al* 1996), these differences may be due to different functions of CCR1 in humans and mice.

Further investigation into the role of CCR1 *in-vivo* was obtained by generating CCR1 null mice. These CCR1 null mice are viable, indicating that CCR1 is dispensable for growth and development and there are also no apparent defects in the haemopoietic and reproductive systems. However, these mice are susceptible to Aspergillus infection, produce a reduced granulomatous response to schistosome egg challenge and reduced pneumonitis in a pancreatitis-induced pneumonitis model (Gao 1997, Gerard 1997), indicating that CCR1 is involved in various inflammatory responses.

The role of CCR1 in growth regulation within the stem cell compartment is not clear. Su *et al* reported that by using anti-CCR-1 antibodies, that human CCR1 is the receptor involved in the inhibition of BFU-E colony formation (Su *et al* 1997), however, Broxmeyer *et al* observed that murine CCR1 is not involved in inhibition of myeloid progenitors but is involved in growth promotion of more mature mouse progenitor cells (Broxmeyer *et al* 1999).

2.6.1.2 CCR3

The human cDNA for CCR3 was first identified by Combadiere *et al* and was identified as an eosinophil chemoattractant receptor, and maybe important in allergic responses such as asthma and antihelminthic host defences as in these conditions eosinophil numbers greatly out weigh the other leukocyte populations. The open reading frame predicts a polypeptide chain 355 amino acids in length and this displays closest homology to CCR-1, 62% identical at the aa level. Human CCR3 can interact with Eotaxin, Eotaxin 2 and 3, RANTES, MCP-3 and 4 and MIP-5 /Leukotactin-1. In contrast, the murine CCR3 receptor has been observed to bind MIP1α and RANTES (Post *et al* 1995, Grimaldi *et al* 1999). CCR3 has been observed to be expressed on eosinophils (Kitaura *et al* 1996), basophils (Uguccioni *et al* 1997), mast cells, Th2 lymphocytes (Sallusto *et al* 1997), dendritic cells and microglial cells. The expression of CCR-3 on human monocytes and macrophages can be further enhanced by treatment with IFN-γ (Hariharan *et al* 1999). At present no CCR-3 knockout mouse has yet to be made available however, eotaxin gene disruption has been performed by two different groups who observed conflicting results. The first group Rotheburg *et al* observed that there was a 40% reduction in airway eosinophillia after ovalbumin challenge (Rothenburg *et al* 1997) and the second group reported that there was no reduction at all (Yang *et al* 1998). Thus although CCR3 and its expression on

eosinophils may be involved in allergic airway pathologies further work is needed to confirm this. Interestingly, a modified form of the chemokine macrophage inflammatory protein 4 (MIP-4), Met-chemokine-β7 (CKβ7), which has been alternatively known as pulmonary and activation-regulated chemokine (PARC), dendritic cell-derived C-C chemokine (DCCK-1), was observed to act as a potent specific antagonist for CCR3 (Nibbs *et al* 2000), further indicating the role of CCR3 in asthma and allergic airway inflammation. CCR3 has been observed to be able to act as an effective co receptor for several neurotropic strains of HIV-1 and thus may be involved in the dementia associated with AIDS (Price and Brew 1988). However, not all HIV-1 strains use CCR3 indicating that additional mechanisms may underlie the ability of HIV-1 to cause neurological impairment (He *et al* 1997, Shieh *et al* 1998).

2.6.1.3 CCR5

Human CCR5 was cloned by Samson *et al* in 1996 and is expressed on dendritic cells, $CD34^+$ HPC, activated memory T cells and monocytes (Samson *et al* 1996, Alkahtib *et al* 1996, Bleul *et al* 1997, Ruiz *et al* 1998). Furthermore, the expression of CCR5 on human monocytes and macrophages can be further enhanced by treatment with IFN-γ (Hariharan *et al* 1999). Outwith the haemopoietic system it has been observed to be expressed in neurones and astrocytes within the CNS, on capillary endothelial cells, epithelium and vascular smooth muscle cells. CCR5 ligands are MIP-1α, RANTES, MIP-1β and MCP-2 and these bind with high affinity, their low affinity ligands are MCP-1, 3 and 4 and eotaxin (Combadiere *et al* 1996, Blanpain *et al* 1999). Mouse CCR5 has a similar ligand profile to the human receptor.

The role of CCR5 *in-vivo* was investigated by homologous recombination and on analysis of these CCR5 null mice it was observed that they develop normally, however, they do appear to have a reduced efficiency at clearing *Listeria* infection indicating a partial defect in macrophage function (Zhou *et al* 1998). Similarly the loss of CCR5 in humans has no obvious deleterious effects however, it does protect these individuals from infection by M-tropic HIV viral strains (Liu *et al* 1996, Benkirane *et al* 1997). A more recent study suggested a possible pathogenic role for CCR5 in multiple sclerosis (MS). T cells from MS patients have an increased level of CCR5 compared to that of healthy individuals. Furthermore, these T cells were

observed to secrete high levels of IFN-γ (Balashov *et al* 1999), which has been previously observed to induce the expression of CCR5 and various CC chemokines in monocytes and macrophages.

2.6.1.4 D6

Human and murine D6 were identified by Nibbs *et al* in 1997 and signalling studies indicated that D6 did not use similar signalling pathways to the other CC chemokines, and thus D6 has not been given a systematic name (Nibbs *et al* 1997a, b). Both proteins display an alteration in the highly conserved DRYLAIVHA motif observed in other chemokine receptors changing it to DKYLEIVHA in both human and murine D6. A further difference in D6 compared to other chemokine receptors is a single amino acid switch from an aspartic acid residue in the second transmembrane domain to asparagine. This aspartic acid residue is conserved in most chemokine receptors and Farzan *et al* observed that on mutating the aspartic acid residue in human CCR5, they can interfere with signalling processes but not ligand binding (Farzan *et al* 1999). Therefore, it is possible that these particular changes in the D6 receptors alter their ability to signal via the normal signalling pathways associated with the chemokine receptors. However, it may be possible to observe signalling through alternative signalling pathways in cell types that naturally express the D6 receptor e.g. lymphatic endothelial cells (Nibbs personal communication). Nibbs *et al* also observed that hD6 does not act as a co receptor with CD4 for entry of M or T tropic isolates of HIV-1 or SIV into human cells however, less abundant viral strains use D6 as a co receptor. The gene has been localised to the gene cluster on human chromosome 3 (Bonini *et al* 1997), human D6 has 71% sequence homology to murine D6 and the receptors display similar receptor ligand binding profiles.

Interestingly, all the ligands that bind D6 have a proline residue at position 2, this may be relevant in light of the discovery of an enzyme Di-peptidyl peptidase IV or CD26, which acts to remove the first two amino acids of proteins that have the N terminal sequence Xaa-Pro, where Xaa is a random amino acid. This has been hypothesised to alter the receptor binding of these chemokines and may also alter the repertoire of receptors that these chemokines interact with (Vanhoof *et al* 1995). Indeed, evidence supporting this hypothesis was reported by Proost *et al* who observed that CD26 truncated RANTES is a chemotactic inhibitor and has increased anti-viral potency,

thus suggesting a role for CD26 in HIV infection and inflammatory processes (Proost *et al* 1998). CD26 digestion has also been observed to reduce the potency of SDF-1 and MIP-1α at inhibiting HIV-1 infection and reduces the chemotactic ability of eotaxin (Proost *et al* 1998, Nibbs *et al* 1999, Struyf *et al* 1999). These studies indicate that the removal of the first two amino acids has varying effects on the activities of different chemokines and it may be possible that CD26 digestion is a method of regulating chemokine interactions with D6.

2.7 Signalling of Chemokine Receptors

The signalling processes involved in the various functions of chemokines are both numerous and extremely complex and are not within the context of this project. Therefore, this section on signalling is only brief and introduces the general concepts involved in chemokine signalling, figure 2.3.

All chemokine receptors are members of the G-protein coupled receptor (GPCR) super family, which also contains rhodopsin receptors, other chemoattractant receptors such as C5a and the classic GPCR, the β adrenergic receptor. Classically the heterotrimeric GTP-binding proteins interact with the 2nd and 3rd intracellular loops of the various chemokine receptors and these G-proteins can either be members of the pertussis toxin-sensitive G_i or insensitive G_q families. Agonist binding to the receptor catalyses the exchange of GTP for the GDP that is bound to the Gα sub-unit and this induces a dissociation /reassociation cycle of Gα and Gβγ sub-units. The GTP bound Gα sub-unit and the Gβγ sub-units then both independently activate downstream effectors such as adenylate cyclase, phospholipase C, PKC and PI-3 Kinase. Furthermore chemokines have been observed to signal through the activation of both the JAK/STAT and MAPK pathways (Myers *et al* 1995, Turner *et al* 1998, Mellado *et al* 1998, Yen *et al* 1997, Wu *et al* 1993, Ganju *et al* 1998a).

In addition, although chemokine receptors lack tyrosine kinase activity, they can also stimulate the phosphorylation of the cytoskeletal protein paxillin (Dutt *et al* 1998), induce the activation of the related focal adhesion tyrosine kinases and various mitogen activated kinases (Ganju *et al* 1998 a, b, Mellado *et al* 1998), through the activation of small guanosine triphosphate-binding proteins of the Ras and Rho

Figure 2.3 An Overview of Chemokine Signalling

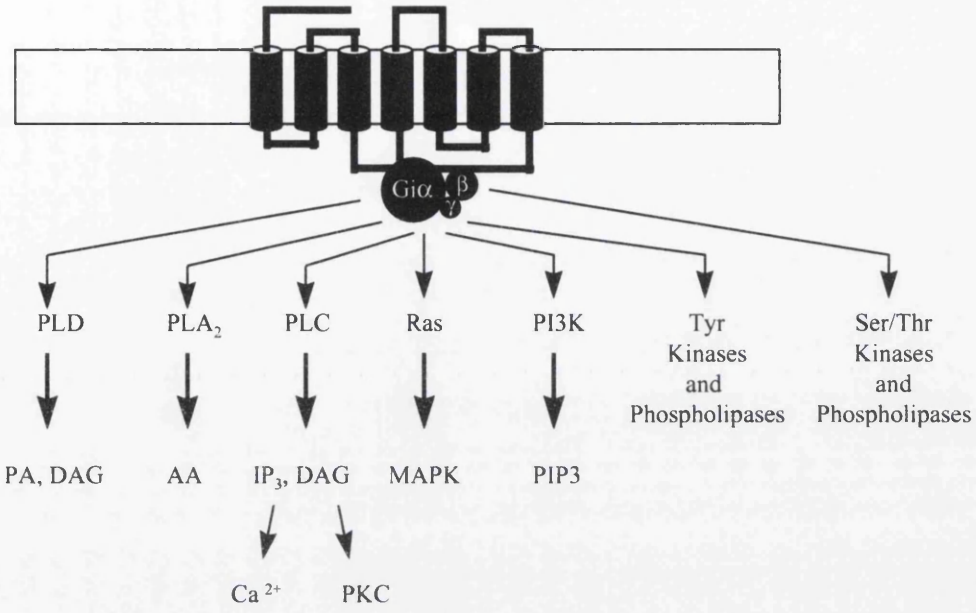


Figure 2.3 : An overview of chemokine signalling

Chemokine receptors are members of the serpentine G-protein coupled family and as indicated above stimulation of the receptor by ligand can induce various signalling pathways.

families. Rho proteins are involved in cell motility through the regulation of actin dependant processes such as membrane ruffling, pseudopodia formation, and assembly of focal adhesion complexes. Indeed, Laudanna reported that the activation of RhoA a low molecular weight G-protein could induce integrin mediated adhesion on stimulation by IL-8 (Laudanna *et al* 1996). This indicates that chemokines can activate signalling pathways using both heterotrimeric and monomeric G-proteins e.g. Ras and Rho. Different cell types express varying combinations of trimeric G protein sub-units, consequently, there are cell type differences in the coupling of G proteins to receptors, which ultimately determine the signal transduction pathway activated in response to the chemokine. Furthermore, variable responses to one chemokine mediated through different receptors can produce differences in Ca^{2+} mobilisation from intracellular versus extracellular stores and differences in downstream signalling events. Indeed, this is the case with the interaction of IL-8 and its receptors CXCR1 and CXCR2 in neutrophils. CXCR-1/ IL-8 activation leads to an increase in Ca^{2+} from intracellular stores and the activation of Phospholipase C and phospholipase D (PLD), which in turn stimulates the release of neutrophilic granules and activates a number of serine/threonine and tyrosine kinases (L-Heureux *et al* 1995). In contrast, IL-8 activation of CXCR-2 stimulates Ca^{2+} influx from outside the cell but does not activate PLD, or induce the release of neutrophilic granules (Damaj *et al* 1996). Thus the control of the signalling potential of a particular chemokine depends not only on the expression of the correct receptor on the surface of the cell, it also depends on the expression of the various types of G-proteins within the various cell types.

2.8 Leukocyte Trafficking

Immunosurveillance and host defence is dependent upon the movement of leukocytes to sites of inflammation, and the subsequent migration of these cells to the lymphoid tissue. Both these processes require the complex interplay between adhesion molecules and chemokines. The maintenance of leukocyte trafficking, whether it be in inflamed tissue or into lymphoid organs, is regulated by a multi step process involving a series of co-ordinated interactions between the leukocytes and the endothelial cells (Butcher 1991, Springer 1994). This process has been postulated to contain four steps: (a) primary adhesion and rolling of leukocytes; (b) activation and arrest; (c) firm adhesion; and (d) diapedesis. A considerable body of *in-vitro* evidence for the role of chemokines in the regulation of leukocyte emigration, from the vascular compartment

to sites of inflammation exists, see review by Adams and Lloyd (Adams and Lloyd 1997).

The majority of research in the chemokine field has indicated that the interaction between chemokines and their receptors induces chemoattraction and activation of myeloid and monocytic cell types whereas, comparatively little is known about chemokines that could interact with the lymphoid cells. However, more recent studies have indicated that there are several chemokines and their receptors that are involved in regulating the movement of lymphocytes and other immune cells. This lead to the chemokines being roughly divided into two functional groups inflammatory chemokines and homeostatic chemokines. The inflammatory chemokines are up regulated by inflammatory or immune stimuli in various cell types such as macrophages and endothelial cells, and function to attract appropriate effector cells into the tissue to respond to the particular pathogen or tissue damage. Whereas, the homeostatic chemokines are constitutively expressed in certain tissues or organs, and are involved in constitutive leukocyte trafficking as well as establishing the cellular compartments within these tissues or organs e.g. secondary lymphoid tissue.

Initial evidence for the role of chemokines in homeostatic trafficking to the compartments of secondary lymph nodes came from knockout studies of a receptor that is normally expressed on B cells, CXCR5. These mice displayed undeveloped B cell follicles in the spleen and Peyers patches, and transfer of these CXCR5 null B cells to wild type mice indicated that these cells could not migrate to splenic follicles. Furthermore, the ligand for CXCR5, B cell attracting chemokine /B lymphocyte chemoattractant (BCA-1/BLC) is expressed in stromal cells of secondary lymphoid organs (Gunn *et al* 1998), indicating a role for this interaction in trafficking of cells in LN. The identification of a further chemokine previously identified as Six-cysteine CC chemokine (6Ckine), Exodus-2, Thymus-derived chemotactic agent (TCA-4) or Secondary lymphoid tissue derived chemokine (SLC), indicated that this ligand was involved in trafficking of dendritic and T cells and the interaction of these cells in the secondary lymphoid tissue (Hedrick *et al* 1997b, Nagira *et al* 1997, Hromas *et al* 1997, Tanabe *et al* 1997). CCR7, the receptor for SLC, is expressed on activated dendritic cells and its expression is higher on naïve compared to activated T cells, further suggesting that the interaction between itself and its ligands may be involved in leukocyte trafficking. The role of SLC in leukocyte trafficking was shown in CCR-7

null mice, where the naive T cells have a reduced ability to enter lymph nodes (Forster *et al* 1999). Furthermore, mice homozygous for a spontaneous mutation *plt* (paucity of lymph node T cells), lack SLC expression in lymphoid organs and have a defective T cell trafficking to lymph nodes (Gunn *et al* 1999). These are only a few examples of how recently identified chemokines are involved in normal haemopoietic cell migration and how this may affect the structure of lymph nodes. An interesting study by Foxman *et al*, indicated that neutrophils that are moving up one chemokine gradient can upon entering another gradient turn and migrate up the new gradient (Foxman *et al* 1997), thus it seems that various leukocytes can respond to complex patterns of chemokine gradients, and these gradients guide these cells to their destinations in inflamed tissue or to lymphoid organs.

2.9 Viruses and Chemokines

Given the important roles of chemokines in diverse immune processes it is not surprising that viruses have exploited chemokine biology. In doing so these viruses can evade the immune system and increase their chances of survival. This phenomenon is emphasised by the large numbers of chemokines, chemokine receptors and general inhibitors and modulators of chemokine action that are encoded within different viral genomes. The chemokine receptors serve as co receptors for two important human pathogens, *Plasmodium vivax* and human immunodeficiency virus (HIV). The malarial parasite uses the DARC receptor to infect erythrocytes whereas, the HIV virus uses CD4 and several chemokine receptors as co factors to infect various cell types and these will be discussed in sections 2.10.

The Kaposi sarcoma associated Herpes virus (KSHV)/ Herpesvirus 8 encodes a number of viral chemokine homologues, vMIP-I, vMIP-II and vMIP-III, which show homology to MIP-1α and have the ability to interact with CCR3 and CCR8 (Moore *et al* 1996, Kledal *et al* 1997, Endres *et al* 1999). Furthermore, Arvanitakis *et al* observed that KSHV also encodes a GPCR that can interact with both CC and CXC chemokines. This KSHC receptor has been observed to display angiogenic properties and more interestingly this receptor is constitutively active and can induce proliferation, without ligand activation, in transfected cells (Bais *et al* 1998, Arvanitakis *et al* 1997). The expression of this receptor has been observed in Kaposi

sarcoma and primary lymphoma and the constitutive activation of this receptor may be involved in the regulation of these conditions.

Human cytomegalovirus (HCMV), β-herpesvirus HHV-6, the Herpes virus saimiri (HVS) and Molluscum contagiosum virus (MCV) types 1 and 2 are further examples of viruses that encode chemokine like receptors and chemokine antagonists (Gao *et al* 1994, Isegawa *et al* 1998, Ahuja and Murphy 1993, Krathwohl *et al* 1997). However, detailed descriptions of these are out with the scope of this project and the reader is referred to a number of recent reviews (McFadden *et al* 1998, Lalani *et al* 1999).

2.10 HIV/AIDS and the Role of Chemokine Receptors

HIV enters target cells by direct fusion of the viral and target cell membranes. This is mediated by the viral envelope protein (Env), which binds to CD4 on the target cell with high affinity. CD4 was observed to allow Env mediated fusion, entry and in human cells but not in non-human cells. Further experiments, performed using cell hybrids supported the hypothesis that a co-factor specific to human cells was involved in HIV fusion, rather than a fusion inhibitor in non-human cells. Thus the concept of co-receptor usage for HIV infection was hypothesised long before their discovery. A number of CC chemokines, RANTES, MIP-1α and MIP-β produced by $CD8^+$ T cells lines, can suppress infection of T cells by the M tropic, but not the T tropic, HIV-1 strains (Cocchi *et al* 1995). This observation indicated that chemokines may determine the susceptibility to HIV-1 and that chemokine receptors may act as co-receptors for the HIV-1 infection. Indeed, Feng *et al* showed that a 7 transmembrane receptor they termed Fusin could act in conjunction with CD4 to aid infection of T cell lines (Feng *et al* 1996). Fusin was subsequently observed to be identical to LESTR/CXCR4, and its ligand SDF-1 was observed to suppress infection by T tropic HIV-1 strains (Bleul *et al* 1996). The discovery of CXCR4 as a co-receptor for HIV infection and the suppressive properties of the CC chemokine RANTES, MIP-1α and MIP-1β focused studies on the possibility that M-tropic HIV strains may use another chemokine receptor as an HIV co receptor. Indeed, several groups subsequently observed that CCR5, which binds RANTES, MIP-1α and MIP-1β, is the co-receptor for M-tropic, but not T tropic, HIV-1 strains (Alkhatib *et al* 1996). Interestingly, it has become evident from the isolation of viral isolates over the progression of the disease,

that HIV isolates change their *in-vivo* properties from M- to T-tropic. These studies indicated that M-tropic (CCR5 utilising) viruses are responsible for infection and that the T-tropic (CXCR4 utilising) viruses may be associated with the rapid $CD4^+$ cell decline and the onset of the symptoms of AIDS (Conner *et al* 1994, Doranz *et al* 1996).

CCR-5 and CXCR4 are the predominant co-receptors used for HIV-1 infection however, more recent studies have also indicated that other CC chemokines, such as MDC and I-309 can also inhibit some diverse strains of HIV-1 infecting T lymphocytes or monocytes. These results suggested that MDC and I-309 receptors, CCR4 and CCR-8 can act as HIV co-receptors (Pal *et al* 1997, Horuk *et al* 1998). As well as CCR-4 and CCR8, there are a large number of alternative co-receptors that have been identified that support virus infection in vitro, these include CCR2b (Doranz *et al* 1996), CCR3 (Choe *et al* 1996), D6 (Choe *et al* 1998), CX_3CR1 (Combadiere *et al* 1998), a number of orphan receptors e.g. GPR15 (Farzan *et al* 1997), STRL33 (Liao *et al* 1997) and even some viral receptors, such as US28 from the human cytomegalovirus (Pleskoff *et al* 1997). Generally these receptors are used inefficiently relative to CCR5 or CXCR4 and their *in-vivo* relevance is uncertain (Ebinger *et al* 1998, Zhang *et al* 1998b).

AIMS

The control of the haemopoietic system is ultimately articulated at the level of stem cell proliferation, and regulation of this proliferation is achieved through a balance of positive and negative regulatory signals. Much has been learned about positive growth molecules, but comparatively little is known about the negative regulators and what mechanisms are involved in their actions. Therefore, the characterisation of how one such negative regulator, Macrophage Inflammatory Protein-1alpha (MIP-1α), exerts its inhibitory effects will not only be central to an improvement in our understanding of haemopoietic regulation but may also shed light on cell cycle control in general. Thus, the aim of this thesis was to investigate the mechanisms involved in the inhibition of stem cell proliferation by the chemokine macrophage inflammatory protein-1 alpha (MIP-1α). This was attempted firstly by using subtractive hybridisation and secondly by examining the effects of growth factor alteration on MIP-1α inhibition of CFU-A colony formation.

CHAPTER 3 : MATERIALS

3.1 Tissue Culture Supplies

Suppliers	Address	
Beatson Institute	Glasgow, UK	Sterile Phosphate Buffered Saline (PBS)
Central Services		Sterile Distilled H_20 (DW)
		Sterile Glassware and Pipettes
		L-Broth
R2 Beatson Institute	Glasgow, UK	L929 and AF-1.19T conditioned medium
Becton Dickinson, UK, Ltd	Plymouth, UK	Falcon tubes 15 and 50 mls
Costar	Cambridge, MA, USA	6 and 24 well culture plates
Difco Laboratories	Detroit, Michigan, USA	Agar Noble
Gelman Sciences	Northampton, UK	Sterile acrodisc syringe filters (0.2 mm & 0.45mm)
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)
		Fischers Medium (10X)
		RPMI Medium (10X)
		200mM Glutamine
		7.5% Sodium Bicarbonate
		Sodium Pyruvate
		Foetal Calf Serum
		Donor Horse Serum
		SLM
		α -Medium
Sigma Chemicals	Poole, UK	Endotoxin-free Dulbecco's PBS
		2-Mercaptoehanol (2mM stock in PBS)
		Donor Horse Serum
		Bovine Serum Albumin,
		RPMI-1640 Medium
Sigma Chemicals	Poole, UK	Dulbecco's MEM

3.2 Plastics

Suppliers	Address	
Becton Dickenson	Oxford, UK	FACSTAR Cell sorter
(Falcon)		Tissue Culture Flasks
		50ml & 15ml Centrifuge Tubes
		5ml, 10ml, 20ml Plastipak Syringes
		1ml, 2ml, 5ml, 10ml, 25ml, 50ml Plastic Pipettes
		Cell Strainers
		3 cm Dishes
		2059 Tubes

3.3 Cytokines and Antibodies

Suppliers	Address	
Beatson Institute, R2	Glasgow, UK	mMIP-1α (PM2) deletion was the form of MIP-1α used in these studies and was as described in Graham *et al* 1994
Dynal	Bromborough, UK	Dynabeads (M-450 sheep anti-rat IgG)
Pharmingen	San Diego, CA,USA	Purified Rat Anti-Mouse Monoclonal Antibodies
		Gr-1
		B220
		Mac-1
		CD4
		CD5
		CD8
		Ter119
		FITC Conjugated Sca-1
		PE Conjugated Thy 1.1
		FITC Conjugated IgG Isotype control
		PE Conjugated IgG Isotype control
		Anti-MIP-1α antibody
R & D Systems	Abingdon, UK	rh & rm GM-CSF
		rh & rm SCF

		rm TGF-β
		rm RANTES
		rh & rm IL-11
		rh & rm IL-6
		rm & rh LIF
		rh M-CSF
		rm MCP-3
		rh HCC-1

3.4 Kits and Columns

Suppliers	Address	
Vector Labs	Peterborough, UK	Photobiotinylation Detection Kit
		Accessory Kit
Invitrogen BV	Leek, Netherlands	cDNA Copy Kit
		Micro-Fast Track mRNA isolation Kit
		RnaseZap (RNase Inhibitor)
Applied Biosystems	Warrington, UK	Gene Amp PCR Kit
		RT-PCR Kit
Pharmacia Biotech Ltd	St.Albans, UK	Microspin S 400 HR Columns
		Ready-To-Go DNA Labelling Kit
		NICK Columns Sephadex G-50 DNA grade
Qiagen Inc.	Chatsworth, CA, USA	Qiagen Plasmid Preparation Kits
		Qiagen Gel Extraction Kits

3.5 Membranes, Paper and X-ray Film

Suppliers	Address	
Amersham International plc.	Amersham, UK	Hybond N Nylon Membrane
Genetics research Ltd	Felstead Dumnow,UK	Saran Wrap
Kodak Scientific Imaging Systems Ltd	Cambridge, UK	X-OMAT AR X-ray film
Schleicher & Schuell	Dassel, Germany	3 MM Whatmann Filter paper
Technical Photo Systems	Cumbernauld, UK	Fuji RX Medical X-ray film
Vernon-Carus, Ltd	Preston, UK	Gauze swabs
Whatmann International Ltd	Maidstone, UK	3 MM Blotting Paper

3.6 Nucleotides, Polynucleotides, DNA Ladders

Suppliers	Address	
Amersham International plc.	Amersham, UK	[α-32P]-dCTP : 3000 Ci/mmol
		[α-32P]-dUTP : 3000 Ci/mmol
Sigma Chemical Co Ltd.	Poole, UK	Salmon Sperm DNA
Gibco Life Technologies	Paisley, UK	1 Kb DNA Ladder
		100 bp DNA Ladder
		DNA Mass Ladder
		RNA ladder

3.7 Plasmids and Inserts

Suppliers	Address	
GE May	LRF, London, UK	PBSmCD34
Dr R Nibbs	Glasgow, UK	pSKmβ-actin
		CCR-1
		CCR-5
		CCR-3
		D6
M Walker	Glasgow, UK	pCR2 GAPDH
Beatson Institute		

3.8 Gels

Suppliers	Address	
Flowgen Instruments Ltd.	Sittingbourne, UK	Agarose
		Low Melting point Agarose (LMP)
Gibco Life Technologies	Paisley, UK	Agarose

3.9 Enzymes and Enzyme Buffers

Suppliers	Address	
Gibco BRL	Paisley, UK	All restriction endonucleases with buffer
		unless stated

NBL Gene Sciences Ltd	Cramlington, UK	T4 Polynucleotide Kinase
		DNA Ligase

3.10 Chemicals

Suppliers	Address	
Boehringer Mannheim	Mannheim,	MOPS
	Germany	
Coulter	Luton, UK	Zapo-globin
DIFCO Laboratories	Michigan, USA	Bactoagar
Fisons Scientific Equipment	Loughborough, UK	Acetic Acid
		Chloroform
		Dimethyl Sulfoxide
		Formaldehyde (38% w/v)
		Formamide
		Glycerol
		Methanol
Gibco BRL	Paisley, UK	Agarose
		TRIzol reagent
		Phenol/Chloroform/Isoamyl alcohol
		(25 : 24 : 1, v/v pH 8)
James Burrough Ltd.	Witham, Essex, UK	Ethanol
Northumbria Biologicals Ltd.	Cramlington, UK	BSA
Nycomed	Sheldon, UK	Animal Nycodenz 1.077g/ml
Raymond A Lamb Lab	London. UK	Napthalene Black
Supplies		
Sigma Chemical Co Ltd.	Poole, Dorset, UK	Ampicillin
		Bromophenol Blue
		Dithriothreitol (DTT)
Sigma Chemical Co Ltd	Poole, Dorset, UK	Ethidium Bromide
		DNA samples (10mg/ml)
		RNA samples (1mg/ml in DEPC treated
		water)
		Fura-2AM
		INT

		Streptavidin
		TRNA
		Tween 20
Thornton and Ross	Huddersfield, UK	Liquid Paraffin
Fishers Scientific UK Ltd	Loughborough, UK	All other chemicals not listed above
Stem Cell Technologies	Vancouver, Canada	Methyl-cellulose (# 3232 -EPO, - CSF)

3.11 Animals

Suppliers	Address	
Harlan Olac Ltd.	Bicester , UK	Female B6D2F1 Mice

3.12 Solutions

3.12.1 Cell Culture Solutions

Alpha Stock	5 Litre pack of α-MEM
	1500 mls DW
	100 mg Gentamycin Sulphate
	50 mls MEM x 100 Vitamins
	Filter Sterilise
Alpha Medium (x2)	25 mls DHS
	21 mls Alpha Stock
	3 mls 7.5% Sodium Bicarbonate
	1 ml L-Glutamine
DMEM	365 mls DW
	50 mls 10x Dulbecco's Medium
	50 mls FCS (10%)
DMEM	25 mls 7.5% Sodium Bicarbonate
	5 mls Sodium Pyruvate
	5 mls L-Glutamine
FDCP-Mix Medium (A4 Medium)	400 mls DW
	45 mls 10x Fischers Medium
	100 mls DHS (20 %)
	6.5 mls 7.5% Sodium Bicarbonate

	4.5 mls L-Glutamine
Fischers Medium	87.5 mls DW
	10 mls 10x Fischers Medium
	1.5 mls 7.5% Sodium Bicarbonate
	1 ml L-Glutamine
PBS/2 % FCS	8 mls FCS
	400 mls PBS
RPMI Medium	331 mls DW
	50 mls 10 x RPMI Medium
	100 mls FCS (20%)
	13.5 mls 7.5% Sodium Bicarbonate
	5 mls L-Glutamine
	0.5 ml β-mercaptoethanol
Special Liquid Medium	Supplemented Modified Eagles Medium
	10 % FCS
	2 mM L-Glutamine
Expansion Medium	290 mls DMEM
	100 mls DHS (25%)
	10 mls Glutamine

3.12.2 Electrophoretic Solutions

Gel Loading Buffer	0.25 % Bromophenol Blue
	40 % w/v Sucrose in DW
1x TE	Tris-HCl pH 8
	1 mM EDTA
50 x TAE	0.4 M Tris Acetate
	0.05 M Sodium Acetate (trihydrate)
	M EDTA

3.12.3 Southern Blotting and Hybridisation Solutions

Denaturation Buffer	1.5 M Sodium Chloride
	0.5 N Sodium Hydroxide
Neutralisation Buffer	1 M Tris (pH 7.4)

	1.5 M Sodium Hydroxide
20 x SSC	3 M Sodium Chloride
	0.3 M Tri-Sodium Citrate
Phosphate Wash Buffer (1)	20 mM Di-Sodium Hydrogen
	Orthophosphate
	5 % SDS
Phosphate Wash Buffer (2)	20 mM Di-Sodium Hydrogen
	Orthophosphate
	1 % SDS
Phosphate Wash Buffer (3)	20 mM Di-Sodium Hydrogen
	Orthophosphate
	0.1 % SDS
Phosphate pre-hybridisation Buffer	0.25 M Sodium Dihydrogen
	Orthophosphate
	7 % SDS, pH 7.2

3.12.4 Northern Blotting and Hybridisation Solutions

50 X Denhardts	1 % (w/v) Ficoll 400
	1 % (w/v) Polyvinylpyrolidone
	1 % (w/v) Bovine Serum Albumin
Denhardts pre-hybridisation Buffer	6 x SSC
	5 x Denhardts
	0.5 % SDS
	50 % Formamide
	10 mg/ml Sonicated ss DNA
DEPC Water	400 µls DEPC
	400 mls DW
	Shake briefly and Autoclave overnight
10 X MOPS	0.2 M MOPS
	50 mM Sodium Acetate
	10 mM Sodium-EDTA
RNA Loading Buffer	50 % Glycerol
	1 x MOPS
	Bromophenol to colour made up in

	DEPC water
Wash Buffer 1	2 x SSC
	0.05 % SDS
Wash Buffer 2	0.1 x SSC
	0.1 % SDS

3.12.5 Calcium Signalling Assay Solutions

	Tyrodes buffer

3.12.6 Bacterial Solutions

Beatson Institute	Glasgow, UK	Luria-Broth
Central Services		
L-Amp Broth		0.5 % Yeast Extract
		1 % Bactotryptone
		1 % Sodium Chloride
SOC		2 % w/v Bactotryptone
		0.5 % w/v Yeast Extract
		0.01 M Sodium Chloride
		0.0025 M Potassium Chloride
		0.01 M Magnesium Chloride
		0.01 M Magnesium Sulphate
		0.02 M Glucose

3.12.7 Subtractive Hybridisation Solutions

	0.1 M Tris pH 9.5
TBBS	0.15 M NaCl
	0.1 % Tween 20
3 x Hybridisation Buffer	50 mM Tris pH 8.3
	10 mM EDTA pH 8.0
	0.3 % SDS
	50 mM Tris pH 8.3
Extraction Buffer	0.5 M NaCl

	5 mM EDTA
	10 mM Hepes
HE	1 mM EDTA

3.13 Primers

RT-PCR Primers	β-actin
	5' TCCATCATGAAGTGTGACGT
	3' TACTCCTGCTTGCTGATCCAC
Dr Nibbs	CCR-3
	5' G 173C
	GCAAGAAATCTCTGTGGTGTTTTAGTT
	3' 17RD
	TTTTCACAGCACGTTTTTAGAGAC
	D6
	5' D65F2
	ACATGCCCACCGTTGCTTC
	3' D632
	GTGCAAGGTGATAAGCACT

CHAPTER 4 : METHODS

4.1 Mice

For all work presented the strain B6D2F1, an F1 hybrid of C57B1/6 (females) and DBA2 (male's) strains, were used. All mice were housed within the animal facility of the Beatson Institute. Female mice were used at age 4-6 weeks for all experimental procedures.

4.2 Maintenance of Cell Lines

All cell lines were obtained from the frozen stocks of the Beatson Institute. The FDCP-Mix cell line is a primitive murine haemopoietic cell line, which is dependent on rm IL-3 for growth (Spooncer *et al* 1986). These cells were maintained in Fischers medium supplemented with 20 % DHS, Glutamine, Sodium Bicarbonate and 10 μg/ml of IL-3. The human megakaryocytic progenitor cell line MO7e is also factor dependant (Hendrie *et al* 1991). These cells were maintained in Fischers medium containing, 10 % FCS, glutamine, sodium bicarbonate and 100 μg/ml rh GM-CSF. THP-1 cells are a human monocytic cell line derived from a patient with acute monocytic leukaemia (Tsuchiya *et al* 1980), these cells were grown in RPMI 1640 medium supplemented with 10 % FCS, glutamine, Sodium Bicarbonate and β-mercaptoethanol. RAW cells are murine monocytic cells which are maintained in special liquid medium (Gibco BRL) supplemented with 10 % FCS and 2 mM glutamine. J772.4 cells are a murine monocytic cell line and are maintained in special liquid medium (Gibco BRL) supplemented with 10 % FCS and 2 mM glutamine. Sub culturing of all cells was performed 2/3 times a week and the cells were seeded at $1x10^5$ cells /ml.

4.3 Haemopoietic Cell Line Assays

4.3.1 FDCP-Mix Plating Assay

FDCP-Mix cells in mid log phase were counted on the CASY-1 counter and resuspended to give a cell concentration of 10^5 cells/ml. The FDCP-Mix cells were then diluted to 10^4 cells /ml. 100 µl of these cells were suspended in α-MEM, supplemented with 20 % DHS, rm IL-3 at 10µg/ml and 0.6 % agar in a total volume of 1 ml. The cells were plated in dishes and were incubated in the presence or absence of various concentrations of MIP-1α (10, 50, 100, 250 and 500 ng/ml) in a humidified atmosphere containing 5 % CO_2, 10 % O_2 and 85 % N_2 for 7 days. The colonies could either be counted on day +7 or alternatively stained for 24 hours with INT and counted on day +8.

4.3.2 MO7e Plating Assay

Factor starved or normal MO7e cells in mid log phase were counted on the CASY-1 counter and resuspended to give a cell concentration of 10^5 cells/ml. They were then diluted to 10^4 cells /ml and 100 µl of these cells were suspended in α-MEM, supplemented with 20 % DHS, rm GM-CSF 100µg/ml and 0.6 % agar to a total volume of 1 ml. The cells were then plated in dishes and incubated in the presence or absence of MIP-1α at 100 ng/ml in a humidified atmosphere containing 5 % CO_2, 10 % O_2 and 85 % N_2 for 7 days. The colonies could either be counted on day +7 or alternatively stained for 24 hours with INT and counted on day +8. For factor starvation, 18 hours prior to the experiment mid log phase MO7e cells were washed and incubated in growth medium without rh GM-CSF.

4.3.3 Calcium Mobilisation Assay

Changes in intracellular Ca^{2+} were measured using the fluorescent label FURA-2AM. THP-1 or FDCP-mix cells were harvested and resuspended to 2-5 x 10^6 cells/ml. FURA-2AM (1mg/ml in DMSO) was added to the cells (1mM) and incubated for 45 minutes at 37 °C in an atmosphere of 5 % CO_2, 10 % O_2 and 85 % N_2. The cells were washed and resuspended in Tyrodes buffer to give 2-5 x10^6 cells/ml. FURA-2

fluorescence emission intensity was measured at 37 °C using a Perkin-Elmer LS-50 fluorimeter with a cell holder and built in magnetic stirrer. The samples were excited at 340 nm with a 10 nm band width and the emission was continuously recorded at 500 nm with a 5 nm bandwidth. FURA-2-loaded cells (2 ml, 2-5 x 10^6cell/ml) were transferred to a 4.5 ml cuvette; $CaCl_2$ was added to 1mM and left to equilibrate for 2 minutes. Recombinant murine MIP-1α was added (20 µl, 100 X final concentration) and the increase in intracellular calcium was noted. After approximately 60 seconds, 20 µl of 5 mM Tween 20 was added to lyse the cells to obtain the maximum fluorescence in 1 mM Ca^{2+} (F max). Once the fluorescence level had stabilised, EGTA (20µl, 1 mM pH 7.2) was added to obtain the background fluorescence (F min). The increase in intracellular calcium was calculated according to the equation $[Ca^{2+}]I$ nmol/L = 224[(F2-Fmin)/(Fmax-F2)] - 224[(F1-Fmin)/(Fmax-F1)], where F1 is the intensity before the agonist addition, F2 is the peak intensity after the agonist addition, F max is the intensity after Tween 20 addition, and F min is the intensity after EGTA chelation, 224nm is the dissociation constant of calcium from FURA-2 at 37°C.

4.4 Enrichment and Analysis of CFU-A Stem Cells

4.4.1 Harvesting of Bone Marrow

4-6 week old female B6D2F-1 mice were sacrificed either by cervical dislocation or by CO_2 asphyxiation. Bone marrow cells were obtained from the femora and tibia of the mice. The bones were first cleaned of muscle tissue using a swab soaked in 70 % ethanol, then crushed in PBS/ 2 % FCS, using a mortar and pestle. After crushing, the cell suspension it was passed through a 70 µm cell strainer and collected in a 50 ml centrifuge tube. After one wash in PBS/ 2 % FCS, the bone marrow cells were resuspended in 5 mls of PBS/ 2 % FCS, to give a single cell suspension, and counted on a cell counter (CASY Counter) after lysing the red blood cells with Zapo-globin.

4.4.2 Enrichment of Lin Negative (Lin^-) Cells

Bone marrow was harvested from 10-20 female B6D2F1 mice as described in 4.41. The bone marrow cells were enriched by density gradient cell separation by layering 3 ml of cell suspension (2.5 x 10^7/ ml) on top of 3 ml Nycodenz mixture (density 1.077g/ ml) and spun at 1000 g for 30 minutes at room temperature. The interface cells were harvested, washed once and resuspended in PBS/ 2 % FCS. These cells were further enriched for lineage negative progenitor content by using indirect immunomagnetic selection to remove mature cells according to previously described methods (Hirayama and Ogawa *et al* 1992). The antibodies used included Gr-1 to remove mature granulocytes, B220 to remove B cells and pre-B cells, Mac-1 to remove monocytes and macrophages, Ter 119 to remove erythroid cells and CD4, CD5 and CD8 to remove T cells. Antibody labelling was performed for 30 minutes on ice and agitated every 10 minutes. The labelled cells were washed three times and re-suspended in PBS/ 2 % FCS, mixed with Dynabeads (M-450 sheep anti -rat IgG) at concentration of 1 x 10^7 beads/ ml of cells and incubated at 4 °C for 30 minutes with end-over-end rotation. The tube containing the cells was then placed in a Dynal MPC-1 magnetic particle concentrator (DYNAL, Great Neck, NY, USA) for 2-3 minutes. The non-rosetted cells were harvested using a pasteur pipette, washed once, re-suspended in PBS/ 2 % FCS and kept on ice for further enrichment by cell sorting. These cells were designated lineage negative. The Lin^- cells were diluted and plated in a CFU-A assay alongside bone marrow cells.

4.4.3 Enrichment of $Sca\text{-}1^+/Lin^-$ Cells

Lineage negative cells were isolated from bone marrow as described in section 4.4.2. Lineage negative cells were incubated with FITC-conjugated Sca-1 antibody, and a separate aliquot was also incubated with an FITC-conjugated IgG isotype control antibody. The cells were then washed three times in PBS/ 2 % FCS, added to a 70 μm cell strainer and resuspended to give a final concentration of $1x10^6$ cells/ml. Cytometric analysis and cell sorting were performed on a FACStar (Becton Dickinson) with an argon-ion laser tuned to 488 nm at power of 200mW. FITC fluorescence was measured through a filter arrangement with peak transmittance at 530 nm. Cells with high FITC labelling compared to that of the isotype control fluorescence were sorted into a 12 x 75 mm round bottom plastic tube containing

PBS/ 2 % FCS, these cells were designated Sca-1^+/Lin^-. These cells were then spun at 1000 g for 10 minutes, resuspended in 100 µls of PBS/ 2 % FCS and counted on a haemocytometer. The cloning efficiency of 100 Sca-1^+/ Lin^- cells was compared to 500 Lin^- cells and $5x10^3$ bone marrow in a CFU-A assay, figure 4.1.

4.4.4 Enrichment of Sca-1^+/Thy1.$1^{lo/-}$/Lin^- Cells

Lineage negative cells were incubated with FITC-conjugated Sca-1 antibody and PE-conjugated Thy-1.1 at a concentration of 1µg/ 1 x 10^6 cells. A separate aliquot was also incubated with two IgG isotype control antibodies, which are FITC-conjugated and PE-conjugated. The cells were then washed three times in PBS/ 2 % FCS, added to a 70 µm cell strainer and resuspended to give a final concentration of $1x10^6$ cells/ ml. Cytometric analysis and cell sorting were performed on a FACStar (Becton Dickinson) with an argon-ion laser tuned to 488 nm at power 200 mW. FITC fluorescence was measured through a filter arrangement with peak transmittance at 530 nm and PE fluorescence was measured through a filter arrangement with peak transmittance at 585 nm. Cells with high FITC labelling and low PE labelling compared to that of the isotype control fluorescence were sorted into a 12 x 75 mm round bottom plastic tube containing PBS/ 2 % FCS. These cells were designated Sca-1^+/ Thy1.$1^{lo/-}$/ Lin^-. These cells were then spun at 1000 g for 10 minutes, resuspended in 100 µl of PBS/ 2 % FCS and counted on a haemocytometer. The CFU-A colony formation of one hundred Sca-1^+/ Thy1.$1^{lo/-}$/ Lin^- cells were compared to that of 500 Lin^-, 100 Sca^+/Lin^- and 100 Sca-1^+/ Thy1.1^+/ Lin^- cells and $5x10^3$ bone marrow cells, figues 4.2.

4.5 Colony Forming Unit -Agar (CFU-A) Assay

The protocol for the CFU-A assay has been described in detail previously (Pragnell *et al* 1988). Briefly, $5x10^3$ bone marrow cells were suspended in α-MEM, supplemented with 25 % DHS and 0.3 % agar to a total volume of 4 mls.

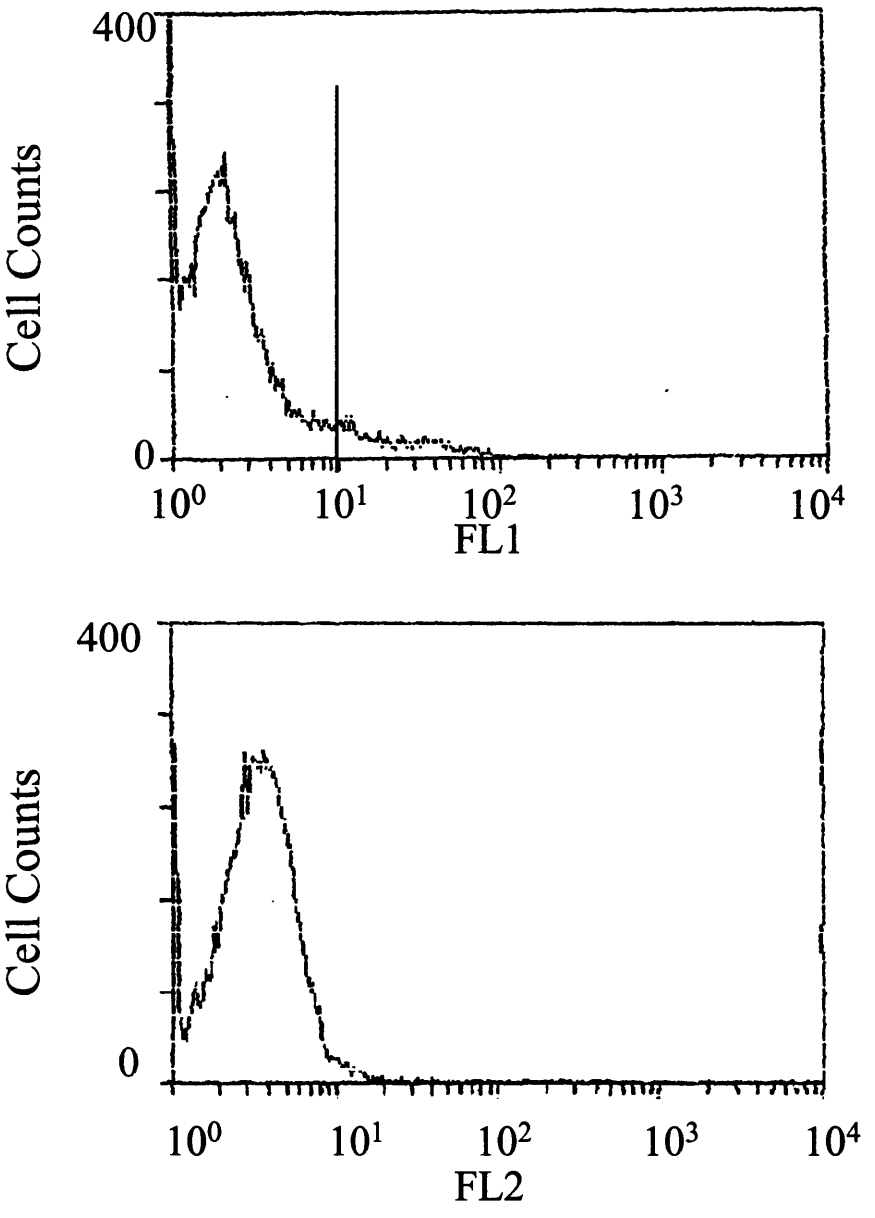


Figure 4.1: Histograms of lin$^-$ cells labelled with Sca-1 FITC antibody or control IgG FITC antibody

The bottom histogram depicts lin$^-$ cells stained with a control IgG antibody. Using this as a comparison, cells that were Sca-1$^+$ and Sca-1$^-$ were selected and are depicted in the top histogram. The Sca-1$^+$ cells are on the right hand side of the line whereas cells that are Sca-1$^-$ are on the left-hand side of the line. These cells are thus sorted and plated in a CFU-A assay.

This suspension was seeded onto a 4 ml feeder layer consisting of 0.6 % agar in the same medium containing either 10 % L929-CM and 10 % AF1-19T-CM or rh M-CSF 6 ng/ml, rm GM-CSF 0.2 ng/ml and 12 ng/ml rm SCF as a source of synergising growth factors. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 11 days. The "cut-off" for colonies to be included as a CFU-A colony was greater than or equal to 2 mm in diameter and this was not varied. This assay detects multi-potential progenitors with proliferative properties in common with CFU-S, and as such offers a means of measuring transient engrafting haemopoietic stem cell populations (Lorimore *et al* 1990, Pragnell *et al* 1988).

4.6 Viability of Cells

10 µl of Sca-1^+/ Thy$1.1^{lo/-}$ / Lin^- cells were spun down at 2000 rpm for 5 minutes in a microfuge. The medium was removed and the cells were resuspended in 2.5 µl of PBS/ 2 % FCS. 7.5 µl of Napthalene black was added and the cells were counted on a haemocytometer. The dead cells were identified by the uptake of the dye.

4.7 Cytospin of CFU-A Colonies

Briefly, $5x10^3$ bone marrow cells were suspended in methyl cellulose (#3230 : -CSF-1, -EPO). This suspension was seeded onto a 1 ml feeder layer, identical to the one described in section 4.5. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 11 days. Using a dissecting microscope five colonies per control and 10 colonies per MIP-1α treated plates were removed and pooled in 400 µl of ice cold PBS. The colonies were washed three times in 1 ml ice cold PBS, and resuspended in a further fresh 400 µl of PBS. The cells were placed in the sample chamber and spun in the cytospin centrifuge at 200 g for 5 minutes. The sample slides were allowed to air dry and were then fixed by immersing in methanol for 2 minutes air dried again and stained with Giemsa.

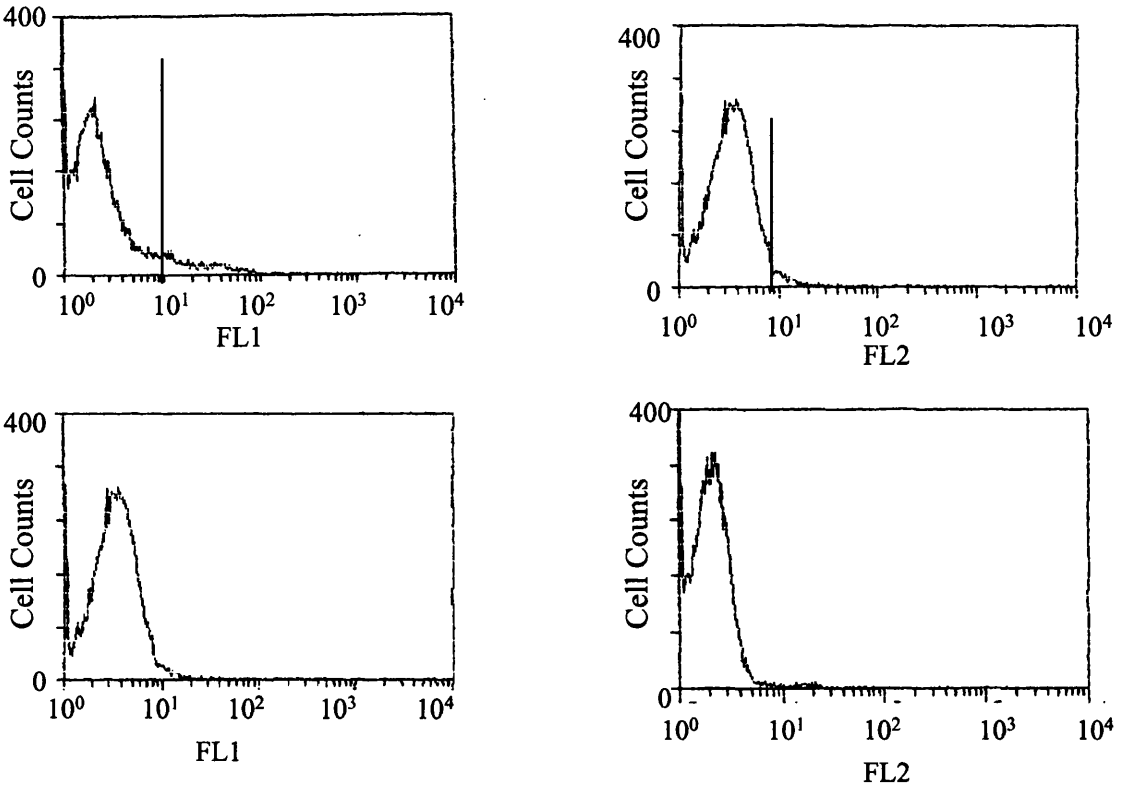


Figure 4.2. Histograms of lin$^-$ cells labelled with Sca-1 FITC antibody or control IgG FITC antibody and Thy1.1 PE antibody or IgG PE control antibody

The bottom histograms depicts lin$^-$ cells stained with a control IgG FITC (FL1) and PE (FL2) antibodies and using this as a comparison the top histograms have gated cells that are Sca-1$^+$ / Thy-1.1 $^{lo/-}$ and Sca-1$^+$./ Thy-1.1 $^+$. These cells are thus sorted and plated in a CFU-A assay.

4.8 Alpha (α) MEM (5 litres)

Alpha medium stock solution was made by adding alpha medium (Gibco 072-2000P 10 litre pack), MEM Vitamin stock 100 x, Gentamycin 200 mg to 3 litres of pre-heated ultra pure water with constant stirring. The media was then filtered through a pre filter, preventing the need to filter through stacked filters of 5, 1.2, 0,8, 0.45 and 0.22 micron pore size. Finally the media was sterilised by passing it through a 0.2 micron filter, aliquoted and frozen at -20 °C

4.9 Conditioned Medium

The L929 (Stanley and Heard 1977) and AF-19T (Franz *et al* 1985) cell lines were grown in roller bottles in SLM/ FCS to half confluence. The spent medium was then removed and replaced with fresh medium and the cultures were allowed to grow for a further three days. The CM was then removed and sterilised by passing it through a 0.45 micron filter and then through a 0.22 micron filter. Finally the CM was aliquoted and stored at -20 °C. In the CFU-A assay, CM from the murine L929 cell line was used as a source of M-CSF and AF1-19T-CM was a source of GM-CSF.

4.10 Derivation of Bone Marrow Macrophages (BMM)

Bone marrow macrophage cells were produced by resuspending bone marrow cells at 5 x 10^4 /ml in a modified MEM supplemented with 25 % DHS and 20 % L929 CM as a source of MCSF. Following incubation for 7 days at 37 °C in a dry atmosphere of 5 % CO_2, the remaining adherent cells consisted of a homogenous (>99%) population of bone marrow-derived macrophages (BMM), as determined by Jarmin *et al.*

4.11 Stimulation of Cell Lines and Bone Marrow Macrophages (BMM)

All reasonable precautions were taken to ensure contamination of experiments with endotoxin/ lipopolysaccharide (LPS) did not occur. This included the purchasing of endotoxin-free recombinant cytokines and PBS, as well as the use of sterile plastic tissue culture pipettes and aerosol plugged pipetteman tips. Bone marrow macrophages were made as in section 4.10. Recombinant SCF was reconstituted according to the manufactures guidelines in endotoxin free PBS/ 0.1 % BSA (v/v).

The monocytic cell lines RAW cells and J774.2 cells can be stimulated to produce MIP-1α by endotoxin. For this reason stem cell factor was reconstituted in endotoxin free PBS. PBS or 100 ng/ml of SCF was added to the cell lines and 250 µl of media was removed at 0, 16, 24 and 48 hours.

4.12 MIP-1α ELISA

MIP-1α protein expression was analysed using a mouse MIP-1α Quantikine M immunoassay from R&D Systems. Briefly, 50 µl of the assay diluent RD1-21 was added to each well in to which a further 50 µl of standard, control or sample was added. This was gently mixed and incubated at room temperature for 2 hours. Each well was then aspirated, washed 5 times with the wash buffer and 100 µl of mMIP-1α conjugate was then added to each well and incubated for 2 hours. After a further aspiration and wash step as above 100 µl of substrate solution was added and this was incubated in the dark for 30 minutes. The reaction was stopped by the addition of 100 µl of stop solution and the optical density of each well was determined within 30 minutes using a micro-plate reader set to 450 nm.

4.13 Neutralising MIP-1α Antibody Experiment

The CFU-A assay was set up as described in section 4.5. Recombinant murine GM-CSF and rh M-CSF were added to the assay at 0.2 and 6 ng/ml respectively with SCF at 1.2, 12 or 120 ng/ml. As well as the growth factors an anti-murine MIP-1α neutralising antibody was added to the CFU-A assay at a concentration of 5 µg/ml. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 11 days and colonies were enumerated on a dissecting microscope. The same assay was performed with the rh M-CSF concentration altered to 0.6, 6 and 60 ng /ml with the rm SCF and rm GM-CSF concentrations staying constant at 12 ng/ml and 0.2 ng/ml.

4.14 Replating Assay

Briefly, 5 x 10^3 bone marrow cells were suspended in 1 ml of methyl cellulose (#3230 : -CSF-1, -EPO). This suspension was seeded onto a 1 ml feeder layer, identical to the one described in section 4.5, the only difference being the addition of various concentrations of SCF, M-CSF or IL-6. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 7 days. Using a dissecting microscope individual colonies were picked from each treatment type and placed in ice cold PBS, 10 in total. Each individual colony was then suspended in α-MEM, supplemented with 25 % DHS and 0.3 % agar to a total volume of 1 ml. This suspension was seeded onto a 1 ml feeder layer, identical to the one described in section 4.5. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 11 days and colonies were enumerated using a dissecting microscope.

4.15 Analysis of Numbers of Cells in CFU-A Colonies

Briefly, 5 x 10^3 bone marrow cells were suspended in methyl cellulose (#3230 : -CSF-1, -EPO). This suspension was seeded onto a 1 ml feeder layer, identical to the one described in section 4.5. Dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 11 days. Using a dissecting microscope five colonies per control and 10 colonies per MIP-1α treated plates were removed and pooled in 400 µl of ice cold PBS. The colonies were washed three times in 1ml ice cold PBS, diluted and stained with methylene blue for counting on a haemocytometer.

4.16 IL-6, IL-11 and LIF CFU-A Assay

Dose responses of all cytokines were performed in the CFU-A assay (0.5, 1, 5, 10, 50 and 100 ng/ml) in the absence or presence of MIP-1α at 1, 10 and 100 ng/ml. The CFU-A assay was set up as described in section 4.5.

4.17 Ex-vivo Expansion

A B6D2F1 mouse was sacrificed either by cervical dislocation or by CO_2 asphyxiation. The femoral bones were cleaned of muscle tissue using a swab soaked in 70 % ethanol. The bone marrow cells were flushed from the bones using a 21-gauge needle containing PBS/ 2% FCS and were counted and diluted to 5 x 10^5 cells/ ml. These cells were then plated in expansion media in non tissue culture grade 3 cm petri dishes in the presence of various single cytokines or combinations of cytokines (SCF 18 ng/ml (KLS from CHO cells), rh IL-11 100 ng/ml, rm MIP-1α 100 ng/ml) Dishes were incubated in a humidified atmosphere containing 5 % CO_2, 10 % O_2 and 85 % N_2 for 6 days. The cells were then harvested from the dishes, washed three times in expansion media and counted. Finally the cells were plated out in a CFU-A assay and incubated as previously described in section 4.5. The CFU-A colonies were scored using a dissecting microscope.

4.18 Photographing Colonies

To facilitate the photography, the colonies were stained with a solution of INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride hydrate). 200 µl of INT (1mg/ml) was placed on each dish and the dishes were incubated in a humidified atmosphere containing 10 % CO_2, 5 % O_2 and 85 % N_2 for 24 hours

4.19 Agar Films

All manipulations with glutaraldehyde were carried out in a fume hood. Briefly, 1 ml of 2.5 % glutaraldehyde solution was added to the CFU-A assays in the 3 cm dish and incubated at room temp for 10 minutes. The top layer of the agar was removed by running the end of a small spatula around the circumference of the top of the agar. The lower layer of agar was then carefully removed into a 9 cm petri dish containing 20 mls distilled water. The dH_20 was changed several times over the next 48 hours to destain each agar film. The agar films were then transferred to a sandwich box containing fresh dH_20 water. Each agar film was removed from the water by placing a glass slide below the film and carefully lifting the film out of the water. The slides were then labelled and a piece of pre wet Whatman chromatography paper (DE81) was placed on top of the film and allowed to dry over night. The filter paper was

removed by running warm water over it and any excess fibers were removed by gently running a finger over the surface of the film. The slides were then placed in a slide rack and this was placed in a glass staining vessel ready to be stained by using May-Grunwald (MG) and Giemsa. MG and Giemsa should be made afresh by adding 71 mls MG to 429 mls of buffer and by adding 6.6 mls of Giemsa to 495.4 mls of buffer. MG was added to the slide rack and incubated for 20 minutes at 37 °C. The MG was removed by running water into the staining vessel and the slides were subsequently stained with Giemsa for 40 minutes at 37°C. Once the stain was removed, the slides were dried and the agar films were mounted with a coverslip.

4.20 Molecular Techniques

4.20.1 Propagation of Plasmid DNA in Bacterial Cells

DH5α competent cells were transformed with the plasmid construct of interest. DH5α cells were removed from -70 °C and thawed on ice for 20 minutes. 20-50 µl aliquots of the cells were dispensed into pre-chilled sterile 2059 falcon tubes and 10-50 ng/ml of plasmid construct was added to the cells gently swirled and incubated on ice for 30 minutes. The cells were then "heat shocked" at 42 °C for 45 seconds exactly, returned to ice for 2 minutes and 90 µl of SOC medium was added. This mixture was shaken at 225 rpm for 1 hour at 37 °C to allow the expression of the ampicillin or tetracycline resistance gene. 100 µl and 50 µl of the transformed cell mixture was spread evenly onto LB Amp /Tetr plates using a glass spreader sterilised by flaming in ethanol. These plates were previously made by adding 7.25g of Bactoagar to 400 ml L-Broth. This mixture was then autoclaved and allowed to cool to 37 °C, 50 µg/ml Amp or Tetr was added to the mixture and 10 mls were poured into a 10 cm petri dish and allowed to set. The plates were left for 5-10 minutes and inverted and incubated at 37 °C overnight.

4.20.2 Growth of Plasmids

A single bacterial colony was plucked from the L-Amp or the L-Tetr plates using a sterile eppendorf tip and added to 10 mls of L-Amp broth plus 50 μg/ml Amp or Tetr in a 2059 falcon tube. This was placed in a shaker and shook at 225 rpm for 16 hours at 37 °C. The mixture was added then to 400 ml of L-Amp or L-Tetr broth in a 2 litre conical flask and incubated under identical conditions for a further 16 hours. The suspension was then transferred to 250 ml centrifuge Sorvall bottle and spun at 3000 rpm for 10 minutes in a Beckman J-6B centrifuge a GS-3 rotor using a Sorvall RC5B super-speed centrifuge. The supernatant was decanted and the pellet was then ready for plasmid preparation.

4.20.3 Plasmid Preparation (Maxi)

A plasmid preparation was performed using the commercially available Qiagen plasmid kit. Briefly, the bacterial pellets were resuspended in 10 mls of re-suspension buffer (P1:100 μg/ml RNase A, 0.05 M Tris/HCL, 0.01 EDTA, pH 8.0) in a 50 ml centrifuge tube (Sorvall instruments). To this 10 mls of lysis buffer (P2: 0.2 M NaOH, 1 % SDS) was added, the solution was mixed gently by inversion and incubated at room temperature for 5 minutes. 10 mls of chilled neutralisation buffer (P3:3 M Potassium Acetate, pH 5.5) was added, mixed and incubated on ice for 20 minutes. Tubes were spun at 4 °C for 30 minutes at 10000 rpm in a Sorvall RC-5B super-speed centrifuge containing SS-34 rotors. The supernatant was then poured through a double layer of gauze swab to remove particulate material. Qiagen-tip 100 columns were equilibrated with 2 x 30 mls of equilibration buffer (QBT: 0.75 M NaCl, 0.05 M MOPS, 15 % Ethanol, 0.15 % Triton X-100, pH 7.0) and the filtered supernatant was applied to the column to purify the DNA. The column was then washed twice with 10 mls of wash buffer (QC: 1 M NaCl, 0.05 M MOPS, 15 % ethanol, pH 7.0) and the DNA was eluted from the column using 15 mls of elution buffer (QF:1.25 M NaCl, 0.05 M Tris/HCL, 15 % ethanol, pH 8.5). The DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 10000 rpm at 4 °C for 30 minutes. The DNA pellet was allowed to air dry and then it was resuspended in 300 μl of dH_20. The DNA was transferred to a 1.5 ml microfuge tube and precipitated by the addition of 0.1 volume 3 M Sodium Acetate pH 5.2 and 2.5

volumes of 100 % ethanol and incubated at -70 °C for 15 minutes. The pellet was washed with 70 % ethanol and spun at 13500 rpm for 5 minutes in a MSE bench top microfuge at 4 °C. To remove the ethanol the pellet was air dried and resuspended in distilled water. The DNA concentration was then measured by spectrophotometry.

4.21 Digestion of DNA

The appropriate amount of DNA to be digested, 10-50µg, was added to a screw capped microfuge tube and 0.1 volumes (of the total reaction volume, normally 100µl) of the appropriate 10 x Reaction buffer was added. Depending upon the restriction enzyme selected, the reaction buffer was chosen to optimise the enzyme activity. When two restriction enzymes are used, a compromise is reached and the buffer is chosen to enable the highest possible efficiency for both enzymes. The restriction enzyme is then added making sure the volume does not exceed 0.1 volumes of the final reaction volume in order to prevent inhibition of the reaction with glycerol within the enzymes. The digest reaction mix was then made up to the desired final volume of the reaction with distilled water, and incubated at 37 °C for 60 minutes. The DNA was either used for downstream applications or store at -20 °C.

4.22 Agarose Gel Electrophoresis

DNA derived from plasmid preps, restriction enzyme digests of plasmid DNA, quantitation of DNA probe fragments and visualisation of PCR products were routinely resolved on a non-denaturing agarose gel. 1 % agarose gels were made by adding 1.5 g agarose to 150 ml 1x TAE and boiled in the microwave. Once the gel had cooled, 10 µl of ethidium bromide (stock 10 mg/ml) was added to the gel to aid visualisation of the DNA. The agarose was then poured into the casting tray and the gel was allowed to set. Loading Buffer (6x) was added to the DNA samples to be analysed to 1/6 of a final volume and the DNA samples were loaded into the gel. A size or mass ladder was always included in the gel. The gels were run at 100 volts until good separation of the molecular weight markers was obtained. Visualisation of ethidium bromide stained DNA bands on a trans-illuminator (λ = 312 nm) and a gel image recorded using an Ampligene imager. For derivation of probe fragments the appropriate bands were excised using a sterile scalpel, for southern blot analysis the image was recorded alongside a fluorescent ruler and excess agarose was trimmed off.

4.23 Isolation of DNA Fragments for Radio-Labelling

Plasmid DNA encoding specific DNA probes was digested with the appropriate restriction enzymes and resolved by agarose gel electrophoresis and the appropriate sized band excised from the gel with a sterile scalpel. The gel fragment was placed in a 1.5 ml screw tap tube and the weight of the agarose was measured. The agarose was removed and DNA eluted, using a QIA quick gel extraction kit. Briefly, 3 volumes (0.1 g of agarose = approximately 100 µl = 1 volume) extraction buffer was added to the excised band in a microfuge tube. Heating to 55 °C for 10 minutes melted the gel and the mixture was added to the QIA quick spin column and spun at 13500 rpm for 1 minute. The flow through was discarded and 750 µl of wash buffer was added to the column and spun at 13500 rpm for 1 minute. A further spin was needed to remove the additional wash buffer. The DNA was eluted into a new 1.5 ml eppendorf by adding 30 µl of TE pH 8 to the centre of the column and spinning as above. The eluted DNA was visualised and quantified by agarose gel electrophoresis, 2-4 µl of DNA was added to a 1 % non-denaturing agarose gel and comparison against a known quantity of low DNA mass ladder.

4.24 RNase Free Environment

To reduce the possibility of degradation of the RNA by RNases several precautions were taken. Disposable, nuclease free, plugged pipetteman tips were used for handling RNA samples. The bench top surfaces and all non-disposable equipment were wiped with RNase-Zap a commercially available solution for inhibiting RNases. DEPC treated sterile water was used for making RNA samples and buffer, ethidium bromide and for resuspending RNA samples.

4.25 Isolation of Total RNA

Total RNA was isolated from the cells using Trizol™ (Gibco, Life Technologies, UK), a commercially available acid guanidinium thiocyanate-phenol-chloroform based extraction mixture (Chomcynski and Sacchi 1987), according to the manufacturer's instructions. Briefly, for RNA isolation from adherent cells in 75cm^2

flasks, growth medium was removed and 10 mls of Trizol™ was added to the flask. 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™ was added to the cell pellet. RNA from adherent and suspension cells was then isolated in an identical way from this point. The flasks were swirled or the tube gently mixed to ensure complete coverage of the full cell area with Trizol™ and left at room temp for 5 minutes. The solution was pipetted up and down several times to ensure complete cell lysis and the solution was then transferred to a 15 ml snap cap Falcon 2059 tube where 2 mls of chloroform was added. Tubes were inverted several times and allowed to stand at room temp for 5 minutes and then spun in a Sorvall RC-5B supercentrifuge at 300 rpm for 15 minutes using HB4 or HB6 swing out rotors. The resulting aqueous phase was then removed to a fresh 15 ml Falcon tube and to this 5 mls of isopropanol was added. The solution was mixed by inversion and left to stand at room temp for 10 minutes. The iso-propanol supernatant was discarded and the pellet was resuspended in 10 mls of 70 % ethanol. The tubes were spun at 3000 rpm for 5 minutes, the ethanol was discarded and the tubes were inverted on the bench for 10 minute to drain off the excess ethanol, the pellets were then air-dried. The RNA was resuspended in 50-100 µl of dH_20 (containing no RNases) and transferred to sterile tubes. The RNA was quantified by adding 3 µl of RNA to 297 µl of distilled water and measuring the OD 260 nm and RNA samples were stored at -70 °C until use.

4.26 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. 5-20 µg of total RNA was added to a screw cap tube and frozen down in the speedi-vac. The pellet was then resuspended in 25 µl of RNA loading buffer and denatured in a water bath at 65 °C for 10 minutes and then placed on ice for 2 minutes to prevent renaturation of RNA strands. 1 µl of ethidium bromide (10 mg/ml) was then added to the RNA and the samples were loaded onto a denaturing agarose gel (1.4 % agarose, 1 x MOPs, 2.2 M formaldehyde and made up to 150 mls with sterile distilled water). Gels were run at a constant 100 volts, until the bromophenol blue band had migrated 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. Gels were then trimmed of excess agarose and northern blotted.

4.27 Detection of Specific DNA Sequences by Southern/Northern Blotting

Both methods are basically the same with only a few minor alterations. For southern blotting the gel was denatured by soaking in denaturation buffer for 3 x 15 minutes with constant agitation. After rinsing with dH_2O, the gel was soaked in neutralisation buffer twice for 15 minutes. Following neutralisation, a glass tank partially filled with 500 ml 10 x SSC and a rectangular piece of glass was raised above the tank to form a platform. A larger rectangular piece of Whatmann 3 MM blotting paper was then soaked in 10 x SSC and placed on the platform with the ends of the paper in the tank of SSC thus forming a wick. The gel was inverted and placed on the 3 MM paper on a solid support over a reservoir of 10 x SSC. The edges of the 3MM paper were under the surface of the 10 x SSC solution. Four pieces of 3 MM paper and a piece of Hybond membrane, were cut to the size of the gel, soaked in 2 X SSC then placed on top of the gel with Hybond membrane adjacent to the gel. Rolling the side of a Pasteur pipette across the membrane before applying the 3MM paper expelled air bubbles. Unwanted, X-ray film was placed around the gel in such a way to ensure that the 20 x SSC from the reservoir would only diffuse through the gel. Paper towels were then stacked on top of the 3 MM paper, a glass plate was placed on top and this was weighed down with a filled 500 ml bottle. This was left overnight to allow capillary transfer of the DNA out of the gel and onto the membrane. The membrane was then carefully removed and washed in 6 x SSC and the DNA was fixed to the membrane by exposure to ultraviolet light (UV Stratalinker).

4.28 Preparation of Radioactive DNA Probes

Probe fragments generated by restriction digest were isolated as before and radiolabelled using a commercially available random priming kit Ready To Go™ according to manufacturer's instructions. Distilled water and 50 ng of DNA inserts were combined to give a total volume of 45 µl and denatured by boiling for 2 minutes and placed on ice for a further 2 minutes and added to Ready To Go ™ tube. All DNA probes used for hybridisation to southern blots were labelled for 15 minutes at 37°C with 5 µl [α-^{32}P]-dCTP, using a random priming kit according to the

manufacturer's instructions. After labelling, the radioactive probe was separated from the un-incorporated ^{32}P-labelled nucleotides by passing through a NICK^{TM} column, previously equilibrated with 1 x TE. The probes were then denatured by boiling for 2 minutes, and placing on ice for 2 minutes before adding them to the blot.

4.29 Pre-Hybridisation and Hybridisation of Southern/Northern Blots

Pre-hybridisation and hybridisation of membranes was performed at 65 °C using 0.2 M Na_2HPO_4/ 7 % SDS pH 7. These steps were performed using bottles and this was carried out in a rotary Hybaid oven. Briefly, the membranes were layered on top of Hybaid mesh and then rolled up and placed in bottles containing 10-20 mls of pre-hybridisation buffer and incubated at 65 °C for at least 1 hour. Following pre-hybridisation, the previously prepared radiolabelled probe was added with fresh hybridisation buffer to the bottle containing the blot and incubated overnight at 65 °C. The above process was performed similarly for northern blots, with the exception being the increase in temperature to 68 °C and the replacement of the pre-hybridisation and hybridisation buffers with a commercially available solution, Express-Hyb from Clonetech.

4.30 Washing Southern/Northern blots and Exposing them to X-ray Film

Southern blots were washed with 2 x SSC/ 0.1% SDS for 30 minutes at room temperature followed by 20 minutes at 65 °C with the above fresh solution and then with 0.5 x SSC/ 1% SDS for 30 minutes at 65 °C. The membranes were monitored and additional washing performed if required, 20 minutes 65 °C 1 x SSC/ 0.1% SDS and finally 10 minutes 0.01 x SSC/ 0.1 % SDS at 65 °C. After the wash steps the membrane is wrapped in Saran Wrap and transferred to an exposure cassette incorporating an intensifying screen. The membranes were exposed to Kodak X-ray film (X-OMATAR) at -70 °C for 4 hours and longer exposures are performed if need be. In the case of northern blots the membranes were washed with 2 x SSC/ 0.1 % SDS for 4 x 10 minutes at room temperature followed by 2 x 20 minutes at 58 °C with 0.5 x SSC/ 1 % SDS. The membranes were monitored and additional washing performed if required e.g. 20 minutes 58 °C 0.01 x SSC/ 0.01 % SDS.

4.31 Stripping of Blots

The blots to be stripped were boiled in 0.1 % SDS for 5 minutes and allowed to cool to room temperature with continous agitation. They were then sealed in Saran Wrap and exposed to X-ray film at -70 °C to be certain that the blot was stripped.

4.32 Phenol/Chloroform Extraction and Ethanol Precipitation

An equal volume of phenol/ chloroform was added to the DNA. The mixture was inverted gently 2-3 times before spinning at 14000 rpm for 10 minutes. The top layer was aspirated and used for the next step in the same way. 1/10 th of the mixture volume of 3 M Sodium Acetate pH 5.2 and 2.5 volumes of absolute ethanol were added to the tube which was then inverted twice and incubated at -70 °C for 15 minutes. The tube was then spun at 14 000 rpm for 15 minutes at 4 °C and the ethanol was carefully removed so as not to dislodge the pellet. The pellet was washed with 1 ml 70 % ethanol and spun at 14000 rpm for a further 5 minutes. Finally the 70 % ethanol was removed, the pellet was air dried and resuspended in distilled water and store at -20 °C until needed.

4.33 Measurement of DNA/RNA Concentration by Spectrophotometry

The absorbance at 260 nm and 280 nm of DNA in solution was measured in a 500 µl cuvette and distilled water was used as a blank. An absorbance of 1 unit (i.e. one optical density (OD) is equivalent to 40 µg/ml of DNA and 50 µg/ml for RNA. The OD 260/ 280 nm ratio gives an estimate of the purity of the DNA/RNA. Pure preparations of DNA/RNA have an OD 260/ 280 nm ratio of 1.8. A 1/100 dilution of the DNA was performed and read in the spectrophotometer.

4.34 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the commercially available Gene Amp RNA PCR Core Kit (Perkin Elmer). The manufacturer's instructions were followed with minor adjustments. Briefly, reverse transcriptase reactions were set up as 20 µl reactions

from a master mix (5 nM MgCl2, 1x PCR buffer II, 1U/ml RNase inhibitor, 2.5 mM Oligo d (T) 16, 1.75 mM random hexamers). 1µl DNase I digested RNA (~1mg/ml) 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 (1mM each of dGTP, dATP, dCTP and dTTP) was added, followed by 1µl of MuLV reverse transcriptase (2.5U/ ml) 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 nM $MgCl_2$, 1 x PCR buffer II, AmpliTaq DNA Polymerase (2.5U/100 µl) and 74 µl added to 20 µl of reverse transcriptase reaction from above. 3 µl each of 5' and 3' primers (200 ng/ml) were then added (see materials section 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; 1 minute at 94 °C, 2 minutes at 60 °C, 2 minutes at 72 °C followed by a soaking at 4 °C. Samples were stored at 4 °C prior to visualisation of 20µl of amplified DNA product, by agarose gel electrophoresis on a 1% agarose gel.

4.35 Subtractive Hybridisation Techniques

4.35.1 Preparation of mRNA from Sca-1$^+$/ Thy1.1 $^{-/lo}$ / Lin$^-$ cells

Sca$^+$/ Thy1.1 $^{lo/-}$/ Lin $^-$ cells were incubated with 100 ng/ml of rm RANTES or rm MIP-1α for 4 hours, then washed in PBS at 4 °C and resuspended in 1ml Lysis Buffer (1 ml Stock Buffer, 20 µl RNase inhibitor). Messenger RNA was isolated from these cells by following the protocol in the Invitrogen mRNA kit. Briefly, the cells were washed in PBS and transferred to a sterile 1.5 ml microfuge tube and spun at 13500 rpm for 15 minutes. The cells were resuspended in 1 ml of lysis buffer by passing them through a sterile syringe fitted with a 18-21 gauge needle and this was repeated three times. The suspension was then incubated for 20 minutes at 45 °C in a shaking water bath and the NaCl concentration was altered to 0.5 M NaCl by adding 63 ml of 5 M NaCl solution and mixed thoroughly. The RNA was then sheared by passing the suspension through a syringe fitted with a 18-21 gauge needle. An Oligo-dT

Cellulose tablet was added to the lysate, the tube was sealed and the tablet was left to swell and dispersed on a rocking platform for 20 minutes. The Oligo-dT was spun at 10000 rpm for 10 minutes and was resuspended in 1.3 mls of binding buffer this was repeated a further twice and the eventual pellet was resuspended in 300 µls of binding buffer. The sample was added to a spin column and spun at 10000 rpm for 10 seconds this was repeated until the entire sample was added to the spin column. The spin column was washed 3 times with binding buffer. The non-polyadenylated RNA was washed off by adding 200 µl of low salt wash buffer and spun as above. The spin column was then placed in a sterile RNase free 1.5 ml microfuge tube and the PolyA$^+$ RNA was eluted by 2 x 100 µl of elution buffer. The RNA was then precipitated and finally resuspended in 10 µl of elution buffer (10 mM Tris pH 7.5) and stored at -70 ^{o}C until needed.

4.35.2 Production of cDNA using Random Primers

Oligo dT priming was used to prime the first strand of cDNA synthesis from isolated Poly A$^+$ RNA populations. Double stranded cDNA was synthesised using the Invitrogen COPY Kit by following the manufacturer's instructions using the Gubler-Hoffman method of cDNA synthesis (Gubler and Hoffman 1983). Briefly, the method uses random priming and AMV reverse transcriptase to convert single stranded RNA into RNA-cDNA hybrids by reverse transcription. The second step utilises the activity of RNase-H to nick the RNA while the RNA-DNA hybrids form. The enzyme E.coli DNA Polymerase 1 then uses these fragments of nicked RNA as primers to synthesise the second strand of cDNA. Any nicks in the double stranded cDNA are repaired with E.coli DNA ligase. These enzymes produce a double stranded cDNA that retains the 3' overhang at the end of the first strand of cDNA and a few RNA nucleotides of RNA sequence at the 5' end of the converted strand. Finally, T4 DNA Polymerase is added to make the double stranded cDNA blunt ended. The cDNA is stored at -70 ^{o}C until needed.

4.35.3 Preparation of cDNA for Subtractive Hybridisation

The double stranded cDNA was digested completely with Alu 1 (this effectively produces smaller cDNA fragments and means that all the cDNA products will be

equally represented when amplified with PCR). Three distinct double-stranded phosphorylated oligodeoxynucleotide linkers were designed and ligated onto each specific cDNA population.

RANTES
5' [GGCCCTGCAGGATCGATCTATAGCG] and
3' [CCGGGACGTCCTAGCTAGATA]

MIP-1α
5'[TAGTCCGAATTCAAGCAAGAGCACA] and
3' [CTTGCTTGAATTCGGACTA]

Bone Marrow
5'[GGCCCTGCAGGATCGATCTATAGCG] and
3'[ATAGATCGATCCTGCAGGGCC]

A Hind III, Not I and a Pst I site have been designed into the three primers respectively. Each linker has one blunt end and one 4-base 3' protruding end. The three cDNA-linker mixtures were electrophoresed through a 2 % low-melting point agarose gel for a short distance to remove the un-ligated linkers. The linker ligated Alu1 cDNA fragments in the size range of 0.2-1Kb were cut from the gel and melted. Linker-ligated cDNA fragments in agarose were amplified directly by PCR. 1 µl of melted agarose was used for 100 µl PCR mixture (94°C, 1 min; 50°C 1 min; 72°C, 1min; Link 4°C Soak; 30 cycles). 10 such PCR reactions were performed for each of the samples. The amplified cDNA fragments were the starting material for subtractive hybridisation and were used for comparative southern blotting analysis with m CD34.

4.35.4 Photo-Biotinylation

10-40 µl of cDNA (0.01 mg/ml in TE) was added to an equal volume of PHOTOPROBE^{Biotin} and mixed gently. For photo coupling, the reaction tube (cap open) was placed in an ice bucket 10 cm below a mercury vapour lamp and illuminated for 5 minutes. A further equal volume of PHOTOPROBE ^{Biotin} was added

and this was again illuminated for 5 minutes under the lamp. The total volume of the reaction was brought to 80 µl with dH_20 and a further 80 µl of Tris pH 9.5 was added this facilitates the removal of the un-incorporated PHOTOPROBE ^{Biotin}. 160 µl of 2-butanol was then added to the mixture and mixed by inversion and this was spun in a micro-centrifuge at 13500 rpm for 5 minutes. The upper butanol phase was discarded and the process was repeated. The biotinylated cDNA was precipitated by the addition of 10 µl 10 M Ammonium Acetate, 2 µl 1 M $MgCl_2$, 1 µl of 20 mg/ml Glycogen and 125 µl of ethanol. This was mixed and incubated at -70 °C for 15 minutes, the biotinylated cDNA was spun down at 13500 rpm for 15 minutes and washed with 70 % ethanol. The pellet was dried and resuspended in dH_20.

4.35.5 Streptavidin Removal of Biotinylated DNA

Briefly, 10 µl of 3 x Extraction Buffer and 4 µl of streptavidin (1mg/ml) was added to 20 µl of biotinylated DNA and left at room temp for 5 minutes. This was performed to allow sufficient time for the biotin/avidin bond to form. 8 µl was removed for later analysis by PCR. An equal volume of TE Saturated Phenol/ Chloroform was added to the mixture and this was vortexed for 30 seconds and spun at 13500rpm for 5 minutes. The top layer was removed to a sterile eppendorf, and to this a further 50 µl of TE_{sat} Phenol/Chloroform was added this again was vortexed 30 seconds and spun at 13500 rpm for 5 minutes. The top layer was removed carefully trying not to transfer any of the bottom layer and this was repeated a further twice. The phenol was extracted from the DNA by adding 50 µl of chloroform, vortexing and spinning as above. A PCR reaction can be set up as a further check for the removal of the photobiotinylated cDNA. Using a master mix consisting of 20 µl Taq Buffer, 40 µl d NTPs (0.05 mM), 20 µl Oligo (2 mM), 2 µl Taq Polymerase, 2 µl Template and 116 µl dH_20. The PCR was set for 2 minutes at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C for 30 cycles followed by a soak of 4 °C.

4.35.6 Subtractive Hybridisation

Subtractive hybridisation was performed according to the protocol from Dr Ged Brady. Briefly, 400 ng of tracer cDNA, made from m MIP-1α stimulated Sca-1^+/ Thy1.1^+/Lin^- cells, 4 µg of photobiotinylated driver cDNA, made from RANTES

stimulated Sca-1$^+$/ Thy1.1$^+$/ Lin$^-$ cells and 5 µg of tRNA were added to a sterile 1.5 ml microfuge tube and the volume was adjusted to 80 µl with HE. The mixture was vortexed for 30 seconds and spun briefly to ensure an even mixture was produced, this was then denatured by boiling for 2 minutes and then incubated on ice for a further 2 minutes. The DNA was precipitated by adding 8 µl 3 M Na Acetate, 200 µl 100 % EtOH, vortexed briefly and chilled on ice for 20 minutes. This was then spun at full speed for 15 minutes and washed with 70 % EtOH and dried for 10 minutes. The pellet was carefully resuspended in 3µl 3 x Hybridisation Buffer and 3µl 5 M NaCl followed by 3 µl 40 % Polyethylene glycol 8000. The sample was then mixed and covered with 70 µl (1-2 drops) oil and transferred to a 0.5 ml microfuge tube. The hybridisation mixture was then placed in a PCR machine and set to run this following program: 5 minutes at 98 °C, 5minutes at 80 °C, 60 minutes at 68 °C then held at 68 °C. Once the program had reached the 68 °C end point 90 µl of extraction buffer (pre-heated to 68 °C to avoid cooling to reduce low stringency hybridisation) was added to the tube. The tube was then briefly spun at 13500 rpm in a bench top micro-centrifuge to ensure all the aqueous layers have combined. The mineral oil was removed from the top of the mixture and the tube was once again spun as above. The bottom 75 µl of hybridisation /extraction mixture was removed to a fresh tube and to this 21 µl of fresh extraction buffer plus 4 µl of 4 mg/ml streptavidin was added, mixed and then incubated at room temperature for 5 minutes. 100 µl of TE saturated phenol/chloroform was added and vortexed for 30 seconds and spun at 13500 rpm at room temperature for 5 minutes. The top three-quarters of the aqueous phase (~75 µl) was removed avoiding the interface and transferred to a fresh new tube. The transferred aqueous phase was extracted with 50 µl of chloroform, vortexed for 30 seconds and spun at 13500 rpm at room temperature for 5 minutes. The above protocol consists of only one round of subtractive hybridisation it was therefore necessary to repeat the above step a further twice. Before doing so 10 µl of the first round of subtracted mixture was removed for further analysis and the remaining 60 µl of the mixture was then put through a further two rounds of subtraction. After a total of three sequential rounds of subtraction the final extracted 60 µl was ethanol precipitated using 2 µg glycogen (2 mg/ml, 2 µl) as a carrier protein, resuspended in 10 µl of TE. 2 µl of each round of subtracted material was amplified using the tracer oligonucleotide as a primer. PCR conditions: 40 seconds 94 °C, 30 seconds 42 °C, 2

minutes 72 °C for 25 cycles. PCR Set up: 53 µl dH_20, 10 µl 10 x Buffer, 7 µl $MgCl_2$, 10 µl Oligo (1-2 mM), 14 µl d NTPs (100 mM), 2 µl template, 2 µl Taq Polymerase.

4.35.7 Cloning, Sequencing and Analysis of Clones from the Subtractive Library

The final round of subtracted material and the PSK-bluescript vector were digested with Not 1 restriction enzyme. After PSK was treated with alkaline phosphatase, the cDNA fragments were ligated into the vector. DH5α competent cells were transformed with PSK-bluescript containing the cDNA inserts from the subtractive hybridisation. The only addition to the method described in 4.20.1 is the addition of IPTG and X-Gal to the Amp plates to provide blue/white selection. The plates were incubated at 37 °C overnight. On the next day any white colonies that formed, were picked and grown up as described in section 4.20.2 and plasmid preps were set up using the Quiagen mini prep kit. Each plasmid was digested to check for insert and then sequenced using the T3 primer. 50 clones were sequenced and were analysed for homologies with known genes and expressed sequence tags (EST's) from non redundant and EST databases using the basic logical alignment search tool (BLAST) search program (Altschul *et al* 1990a, b).

CHAPTER 5

RESULTS 1 : Enrichment of CFU-A stem cells and Subtractive hybridisation

5.1 Introduction

The primary aim of this work was to attempt to examine the molecular mechanisms involved in the inhibition of CFU-A stem cells by the chemokine macrophage inflammatory protein- 1 alpha (MIP-1α). Over the years there has been an increasing number of studies examining how positive growth factors affect cell proliferation. Although there are now more studies examining how negative regulators such as MIP-1α and TGF-β exert their function upon cell proliferation, comparatively little is known about how these negative regulators work. In an attempt to address this, a subtractive hybridisation method was used to examine how MIP-1α exerts its inhibitory function on the transiently engrafting stem cells.

The heterogeneous nature of bone marrow cells and the very low incidence of haemopoietic stem cells have hampered studies of the mechanisms of haemopoietic regulation. The incidence of long term repopulating stem cells (LTRSC) has been estimated to be approximately $1x10^4$ nucleated cells and the estimates vary depending on the assay techniques. Several investigators have employed both physical and immunological techniques in an attempt to enrich haemopoietic stem cells. These techniques have included, density gradient cell separation, centrifugal elutriation, immunomagnetic separation, immune adherence (panning) and fluorescence-activated cell sorting (FACS). The difficulty of enriching such a small stem cell population prompted us to initially attempt to find a cell line that not only displays stem cell characteristics but also can be inhibited by MIP-1α. There are a number of primitive haemopoietic stem cell lines available and our group has examined the effects of MIP-1α on two of these cells. The first cell line examined was a factor dependant cell line, Factor Dependant Cell Paterson- Mix (FDCP-Mix). FDCP-Mix cells were isolated from long term bone marrow cultures infected with a retrovirus carrying the src oncogene (Spooncer *et al* 1986). FDCP-Mix cells are unlike most other growth factor dependant haemopoietic cell lines in that they retain normal karyotype, they are

non leukaemic and the rate of growth factor independent clones is less than 1 in 100 million (Heberlein *et al* 1990). These FDCP-Mix cells are multipotent and are dependent on interleukin-3 (IL3) for growth and survival as the removal of IL-3 from these cells induces apoptosis within 24 hours (Williams *et al* 1990a). One attribute that distinguishes FDCP-Mix cells from other haemopoietic cell lines is their potential to differentiate along multiple haemopoietic lineages in response to regulatory molecules. The addition of IL-3, GMCSF and G-CSF can induce differentiation down the granulocyte lineage and culturing of FDCP-Mix cells with M-CSF or EPO, in the presence of IL-3, can induce macrophage or erythrocyte production respectively. On co-culturing FDCP-Mix cells with bone marrow stromal cells, the FDCP-Mix cells attach and grow on the stroma and undergo multi-lineage differentiation (Spooncer *et al* 1986, Heyworth *et al* 1990). It has also been observed that FDCP-Mix cells have similar properties to CFU-S stem cells in that they have the ability to produce colonies on the surface of spleens of irradiated mice following transplantation. The proliferation of CFU-S stem cells and their *in-vitro* counterparts, the CFU-A stem cells, can be inhibited by MIP-1α. Therefore the similarities between the FDCP-Mix cells and the CFU-A/S stem cells makes this cell line an ideal candidate to examine the effects of MIP-1α.

5.2 The Effect of MIP-1α on FDCP-Mix Colony Formation

In the first experiment, the FDCP-Mix colony growth potential was examined in the presence or absence of increasing concentrations of MIP-1α in soft agar assays. One thousand FDCP-Mix cells were incubated in the presence of varying concentrations of MIP-1α for 7 days at 37 °C 5% CO_2. An average of 60 FDCP-Mix colonies were produced after 7 days, FDCP-Mix colonies were defined as having between 20-40 cells in a tight cluster of cells (figure 5.1). This represents a cloning efficiency of 6 % within the assay and this compares favourably with previously reported results. Indeed, Heyworth *et al* observed a cloning efficiency of 3.5- 5.3 %, and the variation was shown to be dependant on the time the cells were incubated in liquid culture before being transferred to the soft agar culture (Heyworth *et al* 1990). The addition of murine MIP-1α (PM2, see materials) for the duration of the assay at 10, 50, 100 and 500 ng/ml had no detectable inhibitory or stimulatory effect on the number of FDCP-Mix colonies in the soft agar assay. However, the addition of 250 ng/ml of

Fig 5.1

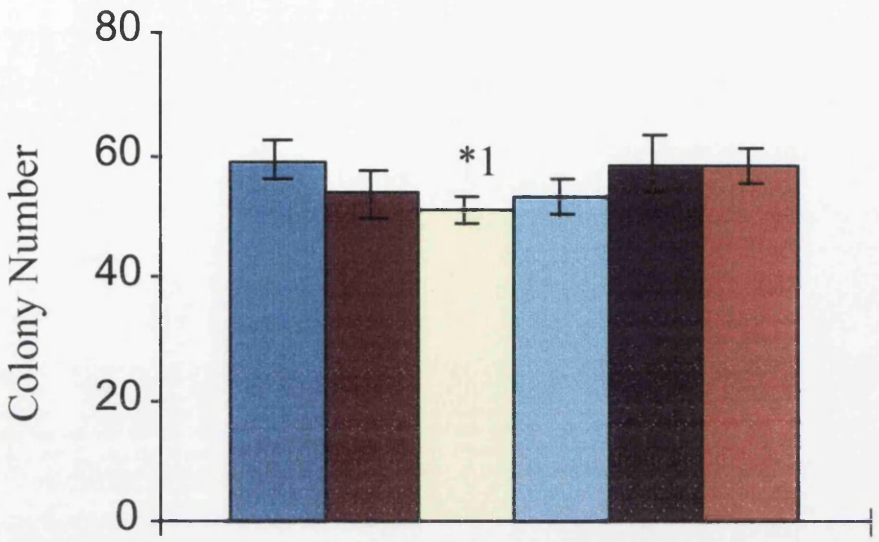


Figure 5.1 Colony Forming assay analysis of the effects of MIP-1α on the production of FDCP-Mix colonies

FDCP-mix cells were cultured for 7 days in soft agar containing rm IL-3 at 10 μg/ml and MIP-1α at 10, 50, 100, 250 and 500 ng/ml. FDCP-Mix colonies were defined as having between 20-40 cells in a tight cluster. The average colony production (+ SEM) is shown for 10 plates. Results represent the mean of three replicate experiments. Students T-test were performed comparing control treatment to each concentration of MIP-1α (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 500 ng/ml MIP-1α
- 250 ng/ml MIP-1α
- 100ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α

MIP-1α produced an 8.6 % reduction in the number of FDCP-Mix colonies, from 60 to 53 colonies. Whilst the inhibition in FDCP-Mix colony formation at 250 ng/ml of MIP-1α is statistically significant ($P < 0.05$), this reduction may be artefactual, as no further inhibition can be observed upon the further increase in MIP-1α concentration. These results are in keeping with previous results within our group, which suggest that MIP-1α does not induce inhibition of FDCP-Mix colony formation. Using [^{3}H] Thymidine, we have been unable to show that MIP-1α affects [^{3}H] Thymidine uptake by these FDCP- Mix cells. These results observed within our group are at odds with the results produced by Heyworth *et al.* They observed that MIP-1α can indeed inhibit the proliferation of FDCP-mix cells in soft agar assays and it can also reduce the uptake of [^{3}H] Thymidine (Heyworth *et al* 1995, Wark *et al* 1998). However, it is possible that the degree of the inhibitory response is dependent on the particular cell isolate used, and this may explain these apparently contradictory results. This inability of MIP-1α to inhibit FDCP-Mix cell proliferation, in our hands, may be explained in a number of ways: (1) There may be a signal transduction problem i.e. the FDCP-Mix cells may not have the ability to produce a signal in response to receptor ligand activation of its various chemokine receptors. (2) The FDCP-Mix cells may not express the appropriate receptor to produce the inhibitory property of MIP-1α.

5.3 Calcium Mobilisation Experiments

The possibility of a signalling abnormality was addressed by analysing the signalling potential of these cells using a calcium mobilisation assay. This type of assay is normally used to study the signalling potential of chemokines in cell lines transfected with chemokine receptors, and is used by several groups to indicate the interaction between a receptor and the signalling mechanisms (Neote *et al* 1993, Myers *et al* 1995, Wu *et al* 1993). Chemokine receptors are members of the 7 transmembrane G protein coupled family of receptors (GPCRs) (Murphy *et al* 2000). On stimulation of the receptor with its ligands, the beta isoform of phospholipase C (PLC-β) catalyses the breakdown of phosphoinositol (4,5) bis-phosphate ($PtdInsP_2$) into two secondary messengers, namely diacylglycerol (DAG) and inositol tris-phosphate ($InsP_3$) (Berridge and Irvine 1989). $InsP_3$ is an intracellular secondary messenger, which can bind to a receptor on the endoplasmic reticulum and trigger the release of calcium

from intracellular stores. Following this, a further increase in intracellular calcium comes as a consequence of the movement of calcium from outside to inside the cell, this may be due to the activation of $InsP_3$ receptors in the plasma membrane (Khan 1992). By using a fluorescent calcium binding molecule such as FURA-2AM, changes in the concentration of intracellular calcium can be followed (Sozzani *et al* 1997). In figure 5.2, the results of two calcium assays can be observed. In assay A, the release of calcium is measured in THP-1 cells upon stimulation with 100 ng/ml of MIP-1α. THP-1 cells are a human monocytic cell line and have a variety of chemokine receptors, principally CCR1 (Nibbs personal communication), expressed on their surface, and are routinely used to analyse chemokine signalling potential. It can be observed that there is an efficient uptake of the fluorescent dye, indicated by a baseline of approximately 208.7, and upon stimulation with 100 ng/ml of MIP-1α a small calcium release shown by the height of the initial spike (230.6) can also be observed.

An estimation of the increase in intracellular calcium concentration was found to be 166.6 nmol/L by using the equation:

$$[Ca^{2+}]i = 224\ [(F_2-F_{min})/\ (F_{max}-F_2)] - 224\ [(F_1-F_{min})/\ (F_{max}-F_1)].$$

Where F_1 is the intensity before the agonist addition, F_2 is the peak intensity after the agonist addition, F_{max} is the intensity after Tween addition, F_{min} is the intensity after EGTA chelation and 224 is the dissociation constant in nM of calcium from FURA-2 at 37 °C. When this experiment is compared to a similar one performed on the FDCP-Mix cells, (figure 5.2B) it can be observed that the FDCP-Mix cells have a lower base line than that of the THP-1 cells i.e. 50.5. On the addition of MIP-1α, there is a small spike in calcium release indicated by the peak and the increase in intensity to 60.1. However, even with a low baseline of fluorescence, the increase in intracellular calcium concentration is equivalent to the THP-1 cells ([Ca]i = 157.5 nmol/L). Both results suggest that neither of these cell lines have a deficiency in signalling through their respective chemokine receptors. However, in both calcium assays the increase in calcium release upon stimulation with the agonist, 100 ng/ml of MIP-1α, is very small and therefore further work is needed to examine the true signalling potential of both of these cell lines.

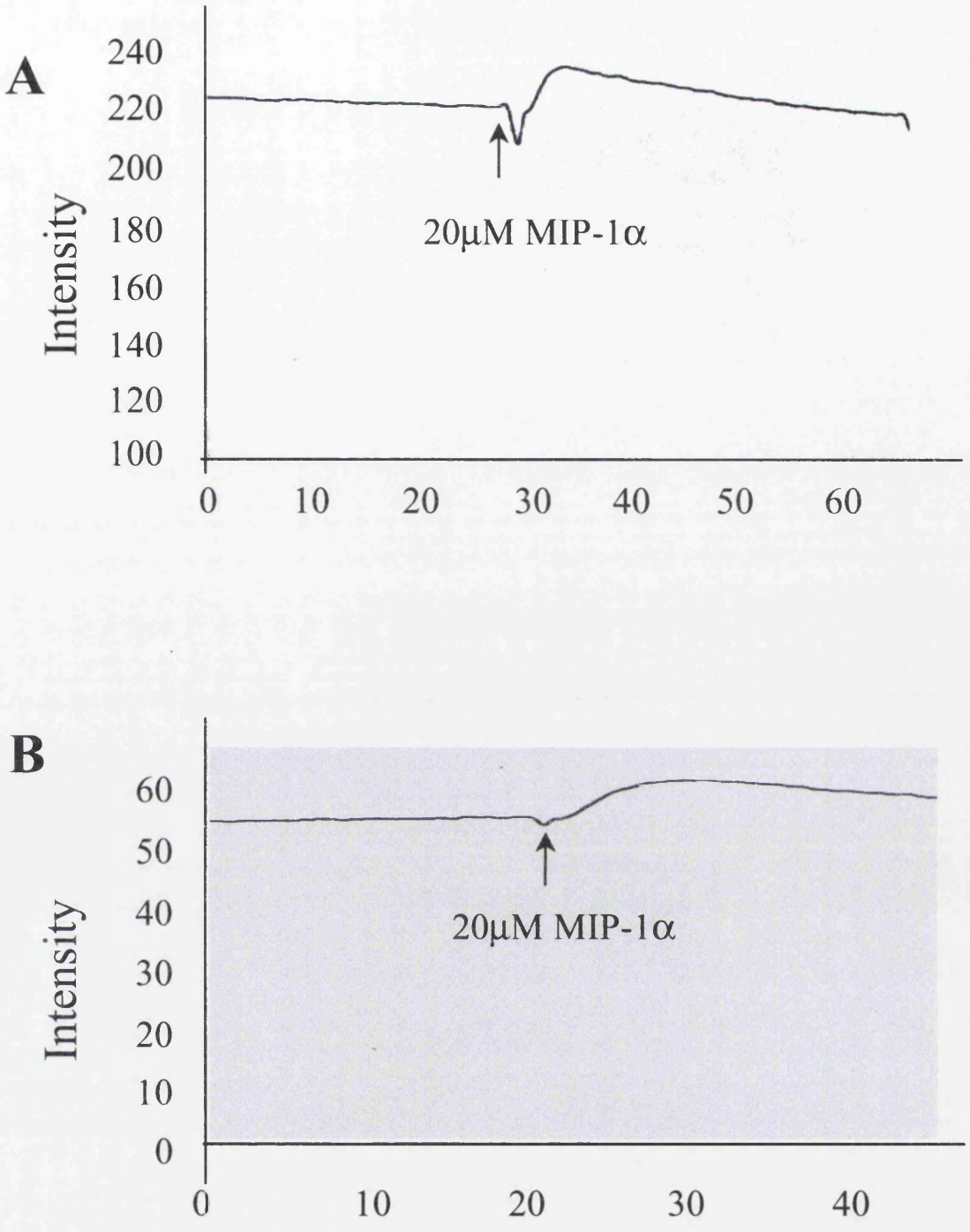


Figure 5.2 Calcium mobilisation assay analysis of the ability of MIP-1α to mobilise calcium in THP-1 and FDCP-Mix cells

Five million cells are assayed in a 2ml cuvette. The fluorescence is allowed to settle and then the 100 ng/ml of MIP-1α is added as indicated by the arrow. This assay is a representative result from three replicate experiments.

A) Shows THP-1 cells as a positive control B) Shows FDCP-Mix cells

Alternatively, it is possible that these cells do not express the unknown MIP-1α inhibitory receptor. Although FDCP-Mix cells have been shown to express CCR1, 3, 5 and D6 (Nibbs personal communication) there is some *in-vitro* data to suggest that these receptors are not involved in the inhibitory activity induced by MIP-1α. However, further studies are needed to clarify the role of these receptors *in-vivo*. Finally, the inability of the FDCP-mix cells to show growth inhibition on incubation with MIP-1α may be due to the dependence on IL-3. The IL-3 induced proliferative drive could be so powerful that any possible inhibition of proliferation by MIP-1α may be hidden. A possible method of getting around this problem could be the removal the growth factor from the cells however, factor starvation of FDCP-Mix cells is not an option as on removal of growth factor these cells die by apoptosis (Williams *et al* 1990a). Therefore, the inability of MIP-1α to inhibit the proliferation of FDCP-Mix cells, indicates that they are not a suitable cell line to use as a model for the inhibitory functions of MIP-1α.

5.4 The Effect of MIP-1α on MO7e Colony Formation

A second haemopoietic cell line, human megakaryocytic progenitor cell line MO7e, was examined for its ability to respond to the inhibitory property of MIP-1α. MO7e cells are also a factor dependent cell line and they depend on rh GM-CSF for survival and growth. It has been reported by Aronica *et al*, that MIP-1α can inhibit proliferation of these cells and that this effect is more pronounced when the cells are factor starved (Aronica *et al* 1995). This factor starvation may reduce the proliferative drive induced by GM-CSF, and thus allow MIP-1α inhibitory effects to be more readily observed. Firstly, the potential of these cells to grow in soft agar was analysed the MO7e cells were cultured for 4 days, and 18 hours prior to plating in agar, the cells were either re-fed or factor starved. The cells were then grown in soft agar in the presence or absence of 100 ng/ml mMIP-1α and 100 ng/ml of rh GM-CSF for 7 days.

Similar to the FDCP-Mix colonies, a MO7e colony was defined as having between 20 and 40 cells clustered together. An average of 110 MO7e colonies were produced when 1 $x10^3$ MO7e cells were plated and this number was reduced to 100 on the addition of 100 ng/ml of MIP-1α, however, this was not significant (figure 5.3A).

Fig 5.3

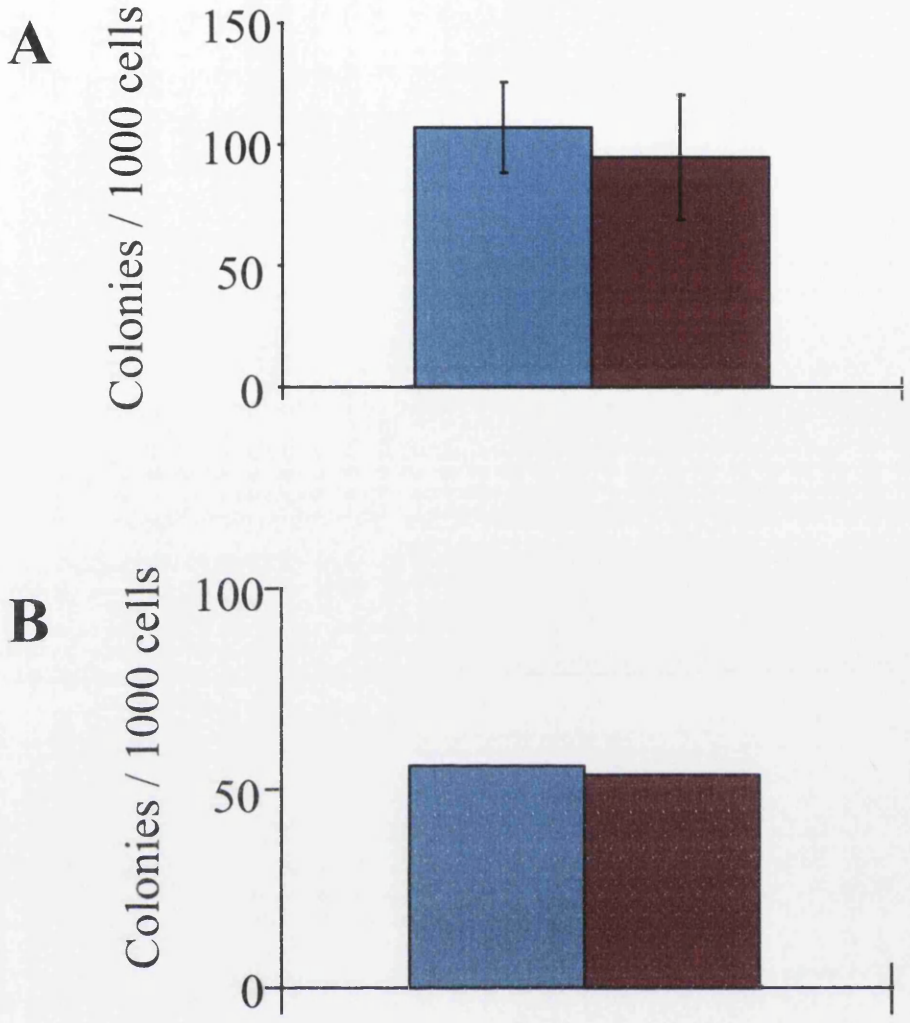


Figure 5.3 Colony Forming assay analysis of the effects of MIP-1α on the production of MO7e cells or factor starved MO7e cells

MO7e cells were cultured for 7 days in soft agar in the presence or absence of rm GM-CSF (100 ng/ml) and MIP-1α 100 ng/ml. MO7e colonies were defined as having between 20-40 cells in a cluster. The average colony production (+ SEM) is shown for 5 plates. Results represent the mean of three replicate experiments. Students T-test were performed (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

A) Normal MO7e cell

- Control
- 100 ng/ml MIP-1α

B) Factor starved MO7e cell

- Control
- 100 ng/ml MIP-1α

This gives a cloning efficiency of 11% and this compares favourably to 10 % found by Mantel and Broxmeyer in 1995 (Mantel *et al* 1995). Furthermore, Broxmeyer *et al* observed that with the inclusion of a factor starvation step, the MO7e cells become more responsive to MIP-1α. However, upon factor starving the MO7e cells and plating 1000 cells in a soft agar assay (figure 5.3B), the overall colony number was reduced from 110 in non factor starved cells to 60 in factor starved cells. No inhibition of MO7e colony formation was observed with the addition of 100 ng/ml of MIP-1α in the factor starved conditions (figure 5.3B). These results are at odds with previously published data by Broxmeyer and Mantel, they demonstrated that MIP-1α at 50 ng/ml could protect MO7e cells from cell death, when exposed to an S-phase cell cytotoxic agent such as ^{3}[H] Thymidine, and that this protective effect increases with the inclusion of a factor starvation step. As with the FDCP-Mix cell line, MIP-1α could not inhibit the formation of MO7e colonies. Therefore, these cells were deemed unsuitable as a model system for the inhibitory action of MIP-1α, and it was decided to pursue the isolation of a highly enriched population of transiently engrafting stem cells.

5.5 Enrichment of CFU-A Stem Cells

5.5.1 Lineage Enrichment of Bone Marrow Cells

The incidence of CFU-A stem cells in normal bone marrow is estimated to be approximately 150-220/10^5 nucleated cells (Lorimore *et al* 1990). The aim is therefore to isolate a population of bone marrow cells that have a high cloning efficiency within the CFU-A assay. Cell separation techniques are based on both size and immunological approaches, these methods rely on diverse cell characteristics, ranging from cell density, cell surface antigen expression and differential sensitivity to cytotoxic agents.

As stated in the introduction, Boyum (1968,1983) originally described a one step centrifugal technique for the isolation of lymphocytes and mononuclear cells. Using this density gradient cell separation technique unfractionated bone marrow from 10-20 B6D2F-1 mice was firstly enriched for mononuclear cells, and then subsequently enriched using an indirect immunomagnetic bead selection, according to a previously described method by Hirayama *et al* (Hirayama and Ogawa 1992). Firstly, the cells

were incubated with a cocktail of monoclonal antibodies specific for cell surface markers expressed on the mature cells which included: B 220 on B cells, CD 4, CD 5 and CD 8 on T cells, Gr-1 and Mac-1 on myelomonocytic cells and Ter 119 on erythroid cells. The labelled cells were then mixed with sheep anti-rat IgG antibody conjugated to immunomagnetic beads and placed in a magnetic particle concentrator. The non-rosetting cells that were recovered from the negative immunomagnetic selection were designated as Lineage negative cells (lin^-), and northern blots confirmed the success of the lineage enrichment (data not shown). To investigate whether the density gradient and immunomagnetic selection could increase the incidence of CFU-A stem cells within this enriched cellular population, the plating efficiency of five hundred lin^- cells were compared to $5x10^3$ unfractionated bone marrow cells in the CFU-A assay (figure 5.4). Unfractionated bone marrow cells produce an average of 10 CFU-A colonies and this concurs with the number of CFU-A stem cells proposed to reside in the bone marrow by Lorimore (Lorimore *et al* 1990). This enrichment technique increased the cloning efficiency from 0.2 %, for unfractionated bone marrow, to 4% for lineage negative enriched cells, a 20 fold enrichment. However, further enrichment of the CFU-A stem cells was required before it was possible to use these cells as a starting cellular population for the subtractive hybridisation.

5.5.2 Enrichment of Lineage Depleted Bone Marrow Cells with Sca-1 Antigen

As is the case with mature cells within the blood the progenitor and stem cells also express specific cell surface markers. The use of these specific markers enables one to isolate a further enriched population of CFU-A stem cells. To enrich for stem cells, several groups have used various cell surface markers that are expressed at various levels on more primitive cells. These included Thy-1.1, Sca-1, *c-kit* and CD34 (Berman *et al* 1985, Spangrude *et al* 1988, Okada *et al* 1991, Morel *et al* 1996 and

Sato *et al* 1999). Sca-1 (stem cell antigen -1) is a glycosyl phosphatidylinositol (GPI)-anchored molecule that is expressed on a number of cell types including: peripheral lymphocytes, thymocytes and hematopoietic precursors and stem cells, as well as on non hematopoietic cells such as fibroblasts, kidney epithelial cells and osteoblasts (van de Rijn *et al* 1989). In this experiment a further enrichment was

Fig 5.4

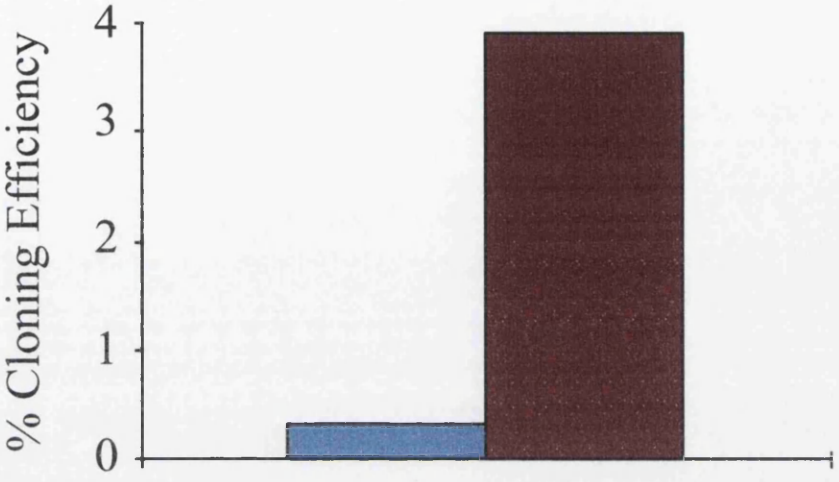


Figure 5.4 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony number by enriching for a subset of bone marrow defined by the lack of mature markers, lineage negative cells (Lin^-).

The cloning efficiency of unfractionated bone marrow and Lin^- cells was calculated when $5x10^3$ unfractionated bone marrow and $5x10^2$ Lin^- cells were cultured for 11 days in the CFU-A assay. CFU-A colonies were defined as colonies greater or equal to 2 mm in size. This assay is a representative result from three replicate experiments. The efficiency of the lineage depletion was checked for mature cell markers via northen blotss (data not shown).

- Cloning efficiency of $5x10^3$ Bone marrow cells
- Cloning efficiency of $5x10^2$ Lineage minus cells

attempted using a Sca-1 monoclonal antibody. After the pre-enrichment step in section 5.51, the lin$^-$ cells were labelled with either a monoclonal antibody to Sca-1 (anti Ly-6A.2/E.1), stained with fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG or with an IgG isotype control antibody stained with FITC. The fluorescent staining of these lin$^-$ cells were compared to that of the isotype control treated cells on the FACS Star. The cells with high fluorescence intensity, as shown in top histogram in figure 4.1, were then sorted as Sca-1$^+$/ Lin$^-$ cells. Furthermore, cells with low to negative Sca-1 staining were also collected and these cells represent a population of cells that are Sca-1$^-$/ Lin$^-$. The CFU-A activity of one hundred Sca-1$^+$ / Lin$^-$ and Sca-1$^-$ / Lin$^-$ cells were then compared with $5x10^3$ unfractionated bone marrow cells and $5x10^2$ lin$^-$ cells. The isolation of Sca-1$^-$/ Lin$^-$ cells did not improve the CFU-A yield however, cells that were positively stained with the Sca-1 antibody, the Sca-1 $^+$/ Lin$^-$ cells, further enriched CFU-A numbers 3 fold producing a cloning efficiency of 12 % (Figure 5.5). Thus the addition of the Sca-1 enrichment step produced an overall 60-fold enrichment from unfractionated bone marrow.

5.5.3 Enrichment of Lineage Depleted Bone Marrow Cells with Sca-1 and Thy-1.1 Antigens

Berman *et al* observed that lineage depleted cells that expressed low but significant levels of a cell surface differentiation antigen Thy-1.1 could be used as an indicator for reconstituting bone marrow stem cells in mice (Berman and Basch 1985). Further evidence indicated that these Thy1.1 $^{lo/-}$ Lin$^-$ cells were observed to be enriched in clonal progenitors for spleen colonies (CFU-S), thymic colonies (CFU-T), pre-B cultures and haemopoietic stem cells (Whitlock *et al* 1987). Spangrude *et al* identified a stem cell population, which is highly enriched in activity in various stem cell assays. These particular cells were depleted of lin$^+$ cells, were positive for the Sca-1 antigen and had low or negative staining for the Thy-1.1 antigen. Spangrude observed that these cells represented approximately 0.05 % in total bone marrow from C57BL/Ka Thy-1.1 and that these cells contained haemopoietic stem cell activity, as limited numbers of these cells could repopulate T, B and myeloid lineages and lead to long term survival of lethally irradiated mice (Spangrude and Johnson 1990). Therefore, the Sca-1$^+$/ Lin$^-$ cells were further enriched for cells that either do not express or cells that express a low level of the Thy-1.1 antigen by using a Thy-1.1

Fig 5.5

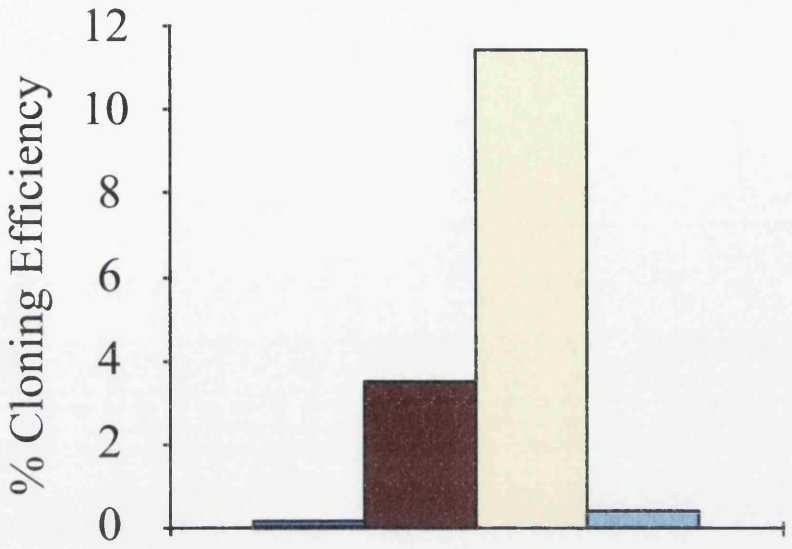


Figure 5.5 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony number by further enriching lineage negative cells (Lin^-) on the expression of Stem cell antigen -1 (Sca-1).

The cloning efficiency of unfractionated bone marrow, Lin^- cells, $Sca\text{-}1^+/ Lin^-$ and $Sca\text{-}1^-/ Lin^-$ cells was calculated for $5x10^3$ unfractionated bone marrow, $5x10^2$ Lin^- cells, $1x10^2$ $Sca\text{-}1^+/ Lin^-$ and $Sca\text{-}1^-/ Lin^-$ cells that were cultured for 11 days in the CFU-A assay. CFU-A colonies were defined as colonies greater or equal to 2 mm in size. This assay is a representative result from three replicate experiments.

- Cloning efficiency of $5x10^3$ Bone marrow cells
- Cloning efficiency of $5x10^2$ Lineage minus cells
- Cloning efficiency of $1x10^2$ $Sca\text{-}1^+/Lin^-$ cells
- Cloning efficiency of $1x10^2$ $Sca\text{-}1^-/Lin^-$ cells

phycoerythrin (PE) conjugated antibody. Thy 1.1 (CD90) is a GPI-anchored membrane glycoprotein of the Ig superfamily which is found on thymocytes, T lymphocytes, haemopoietic stem cells and some neurones (Ritter *et al* 1983). The lineage depleted (lin^-) cells were incubated either with IgG isotype control antibodies stained with FITC and PE, or an IgG monoclonal antibody to Sca-1 (anti Ly-6A.2/E.1), stained with FITC and a monoclonal antibody to Thy-1.1, stained with PE conjugated goat anti-rat IgG. The fluorescent staining of these cells were compared to that of the isotype control treated lin^- cells on the FACS Star, and cells that were Sca-1^+ / Thy1.1 $^{lo/-}$ and Sca-1^+ / Thy1.1 $^+$ were collected (figure 4.2). The ability of these purified cellular populations to form CFU-A colonies was examined and the results can be observed in figure 5.6. One hundred Sca-1^+ / Thy1.1 $^+$ / Lin^- cells did not increase the cloning efficiency over and above that displayed by Sca-1^+ / Lin^- cells within the CFU-A assay. However, the addition of 100 Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin^- cells to the CFU-A assay produced an average of 17.67 CFU-A colonies, which represents a cloning efficiency of 17.67 %. The cell sorting process leads to a reduction in cell viability therefore, to examine the actual cloning efficiency of the Sca-1^+ / Thy1.1 $^+$ / Lin^- cells the viability was taken into account, this was performed by examining the uptake of a viability indicating dye, Napthalene black, by the enriched population cells. From Table 1, it can be observed that 19.26 % of the Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin^- cells took up the dye therefore within the enriched population there are only 80.74% of viable cells. This means that the actual cloning efficiency of these Sca-1^+ / Thy1.1 $^{-/lo}$ / Lin^- cells within the CFU-A assay is 21.98 %, and this produces a total enrichment factor of 109.9. This enrichment process was repeated and a cloning efficiency of 22 % was routinely obtained.

5.6 Inhibition of CFU-A Colony Formation of Sca-1^+ / Thy-1.1 ^{lo} / Lin^- Enriched Cells

To confirm that there was no loss of inhibitory response in these cells the inhibitory effect of MIP-1α was examined. One hundred Sca-1^+ / Thy-1.1 ^{lo} / Lin^- cells were plated in the presence or absence of 100 ng/ml of MIP-1α in a CFU-A assay. In figure 5.7, it can be observed that the addition of MIP-1α reduces CFU-A colony numbers from 17.56 to 3.55 a reduction of 79.88 %.

Fig 5.6

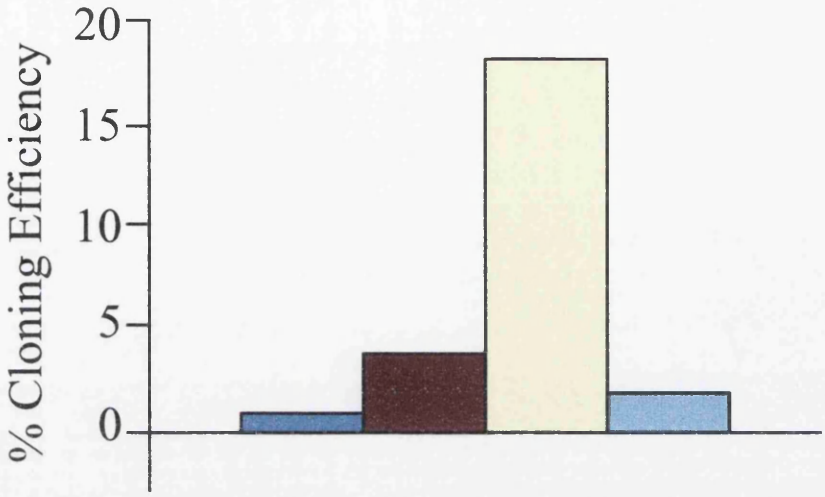


Figure 5.6 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony number by further enriching Sca-1^{+}/Lin^{-} cells on the expression of Thy-1 antigen.

The cloning efficiency of unfractionated bone marrow, Lin^{-} cells, Sca-1^{+}/Thy-1.1 ^{lo} /Lin^{-} and Sca-1^{+}/ Thy1.1 $^{+}$/ Lin^{-} cells was calculated when 5x10^{3} un-fractionated bone marrow, 5x10^{2} Lin^{-} cells, 1x10^{2} Sca-1^{+}/Thy-1.1 ^{lo} / Lin^{-} and Sca-1^{+}/ Thy1.1 $^{+}$/ Lin^{-} cells were cultured for 11 days in the CFU-A assay. CFU-A colonies were defined as colonies greater than or equal to 2 mm in size. This assay is a representative result from three replicate experiments.

- Cloning efficiency of 5x10^{3} Bone marrow cells
- Cloning efficiency of 5x10^{2} Lineage minus cells
- Cloning efficiency of 1x10^{2} Sca-1^{+}/Thy-1.1 $^{lo/-}$ /Lin^{-} cells
- Cloning efficiency of 1x10^{2} Sca-1^{-}/Thy-1.1 $^{+}$ / Lin^{-} cells

Table 1

Test number	Number of cells that do not take up the dye	Number of cells that take up the dye	Total number of cells	% Viability
1	250	49	299	83.61
2	265	77	342	77.48
3	265	60	325	81.54
Average	260	62	322	80.74

Table 1 Analysis of viability of the Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells by the exclusion or uptake of Napthalene Black

10 µl of Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells were spun down and resuspended in 7.5µl of PBS to this 2.5µl of Napthalene Black was added and the cells were counted on a haemocytometer. The cells that took up the dye were counted as dead cells.

5.7 Suitability of the Sca-1$^+$ / Thy1.1 $^{lo/-}$ / Lin$^-$ Cells as the Source for the Subtractive Hybridisation

The overall enrichment protocol reduced the total numbers of cells from 1.8 x10^8 in unfractionated bone marrow to 2-5 x10^4 in the final Sca-1$^+$ / Thy-1.1 ^{lo} / Lin$^-$ population, these cells are highly enriched for CFU-A stem cells and MIP-1α can almost completely inhibit the proliferation of these cells. These enriched cells produce a cloning efficiency of 22 % within the CFU-A assay, this means that there may be a possibility of contamination of the subtractive hybridisation from the other 78 % of the cells that do not grow in the CFU-A assay. Morphological analysis of the residual 78 % of cells suggests that there is no evidence of a substantial contribution of mature cells to the enriched population and, the main constituent cell type of the enriched population was identified as blast cells (Table 2). These cells are primitive haemopoietic cells and characteristically display a high nuclear to cytoplasmic ratio, which can be observed in figure 5.8. As indicated by the cloning efficiency the majority of these blast cells do not produce colonies in the CFU-A assay suggesting that they may be out-with the phenotypic window which scores in the CFU-A assay (Table 2). Therefore, these particular blast cells are not CFU-A stem cells and as MIP-1α can only inhibit CFU-S/A stem cells within the stem cell compartment it is unlikely that there will be an inhibitory signal induced in any of these cells within the finally enriched cellular population. The data in figure 5.9 provides further evidence that this cell population is indeed enriched for stem cells. Southern blot analysis (see methods section 4.27) of the abundance of α-actin, a house keeping gene, and CD34, a known stem cell marker, in the cDNA from Sca-1$^+$ / Thy1.1 $^{lo/-}$ / Lin$^-$ population was compared to that of cDNA from unfractionated bone marrow cells. There is an increase in CD34 expression within the cDNA from the Sca-1$^+$ / Thy1.1 $^{lo/-}$ / Lin$^-$ cells compared to that of the bone marrow again indicating that these cells are primitive haemopoietic cells.

Fig 5.7

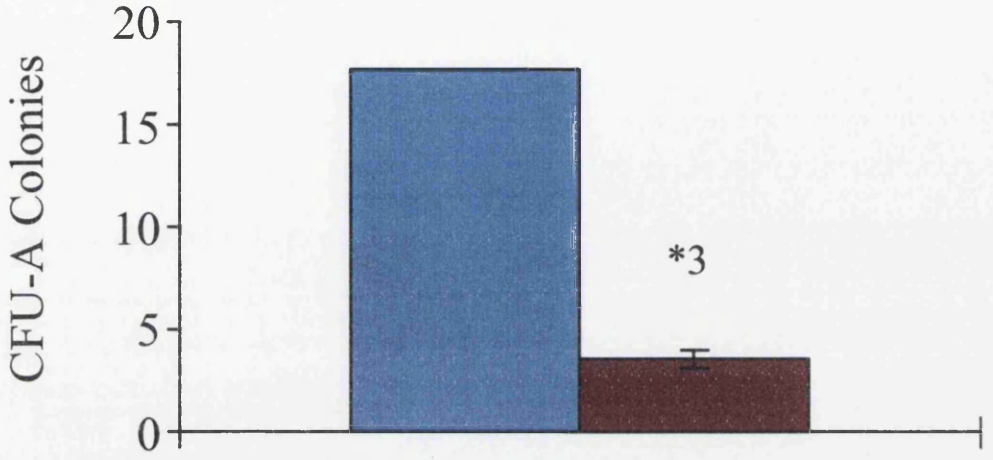


Figure 5.7 Colony Forming Unit Agar (CFU-A) analysis of the effect of 100 ng/ml of MIP-1α on the formation of the CFU-A colonies from Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} enriched cells.

Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} were cultured for 11 days in the presence of 100 ng/ml of MIP-1α in the CFU-A assay. CFU-A colonies were defined as colonies greater or equal to 2 mm in size. The average colony production (+ SEM) is shown for 5 plates. Results represent the mean of three replicate experiments. Students T-test were performed
(* = $P < 0.001$)

- 1x10^{2} Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells
- 1x10^{2} Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells plus 100 ng/ml of MIP-1α

Table 2

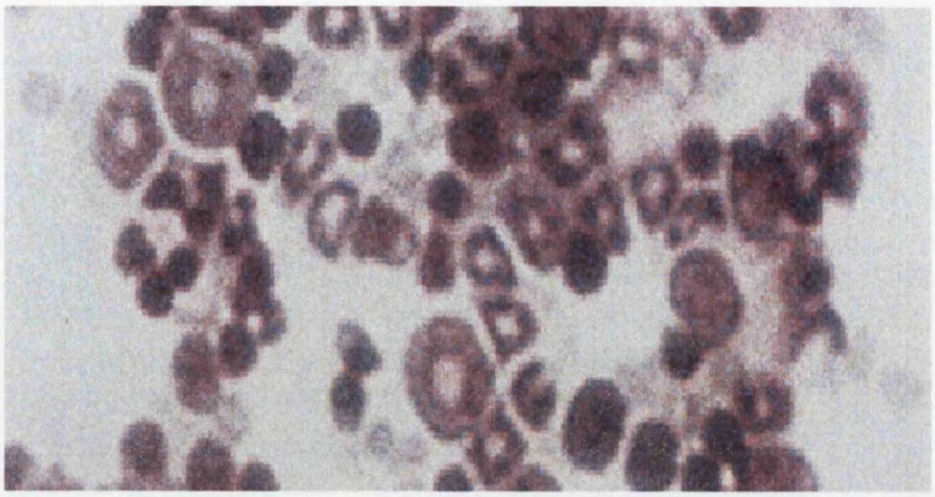
Cell Type	% of cells in enriched population
Blast cells	87.5
Primitive Monocytoid Cells	9.0
Primitive Myeloid Cells	3.5
Mature cells	0

Table 2 Cell morphological analysis of Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells

Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells were isolated as described in the materials and methods and cytospins were prepared. The morphology of 500 cells was examined.

Fig 5.8

A



B

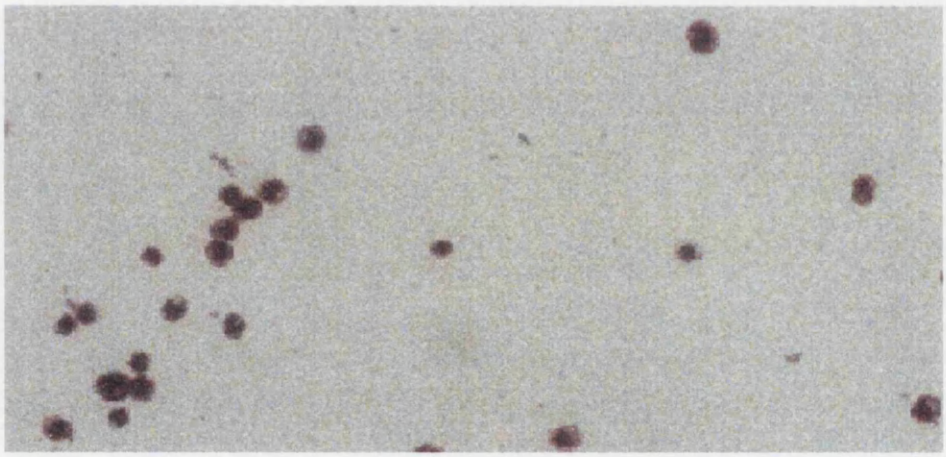


Figure 5.8 Photographic analysis of the morphology of unfractionated bone marrow, lineage depleted cells and Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-}

Bone marrow, lineage depleted and Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} were isolated and cytospins were prepared and photographed.

A) Shows the cell morphology of un-fractionated bone marrow
B) Shows the cell morphology of Sca-1^{+}/ Thy-1.1 ^{lo}/Lin^{-}

Fig 5.9

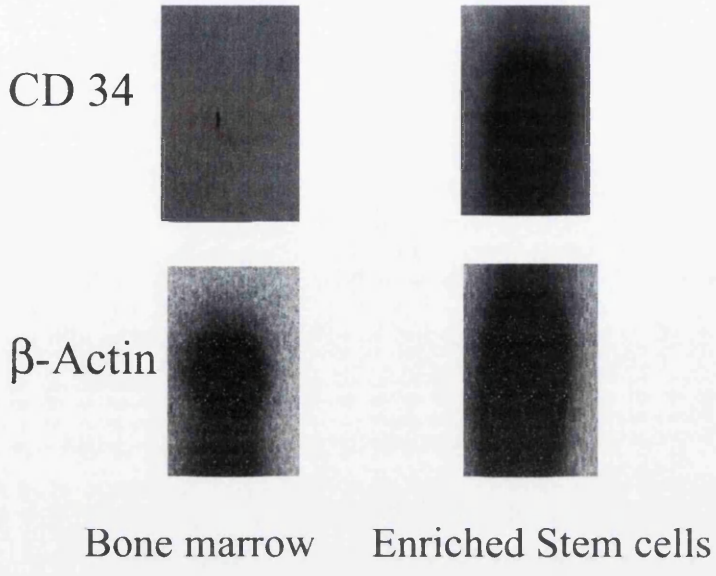


Figure 5.9 Southern Blot Analysis of mCD34 Expression on Bone Marrow and Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} Cells

The top row shows a blot hybridised with a probe for murine CD34 and the bottom panel shows the same blot stripped and reprobed for β-actin. Column 1 is cDNA made from normal bone marrow and column 2 is cDNA from Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells.

As MIP-1α is a member of the C-C chemokine family and has functions other than inhibition of CFU-A/S stem cells, e.g. induction of chemotactic responses on a variety of blood cells, there is a concern over the possibility that signals other than the inhibitory signal produced by MIP-1α may also be transduced in these Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin^- cells and therefore, may be detected by the subtractive hybridisation. In an attempt to overcome the possible problem, the cDNA made from RANTES (regulated upon activation normal T expressed and secreted) treated Sca-1^+ / Thy1.1 $^{lo/-}$/ Lin^- cells was used as subtractive partner. RANTES treated cells were used as subtractive partner because RANTES is also a member of the C-C chemokine family and like MIP-1α it also demonstrates chemotactic responses on a variety of mature blood cells (Rot *et al* 1992). Furthermore, no murine chemokine receptor has been identified that exclusively binds either RANTES or MIP-1α and both chemokines have the ability to bind to the same receptors namely CCR 1, 3, 5 and D6. Therefore, the possible carry over of pro-inflammatory signals contaminating the subtractive hybridisation will be kept to a minimum by using RANTES treated cells as the subtractive partner. As well as these properties RANTES has a further advantage in that it does not inhibit stem cell proliferation and this means that the final cDNA isolated from the subtraction should be relatively specific for the inhibitory effect on CFU-A stem cells by MIP-1α.

5.8 Subtractive Hybridisation

Cellular differentiation and developmental processes are associated with corresponding changes in messenger RNA within a particular cell type. Messages for genes relevant to specific developmental processes are thought to alter their abundance due to induction or repression of transcription. Several groups including ours have used subtractive hybridisation as a method for examining the alteration in gene expression in other situations (Wang and Brown 1998, Brady and Iscove 1993, Baird *et al* 1999). Subtractive hybridisation was used here to examine the induction and repression of gene transcripts that may be involved in MIP-1α inhibition of CFU-A stem cells. However, it is necessary to realise that induction or repression of transcription may not be the only level of control used by MIP-1α to inhibit the CFU-A stem cells.

Indeed, phosphorylation or dephosphorylation of various cellular proteins may be an alternative means of control involved in MIP-1α induced inhibition of CFU-A stem cells. However, these types of studies on stem cells are difficult to perform due to the limited numbers of stem cells and the inability to produce a homogenous stem cell population.

Therefore, although it is accepted that other controlling pathways may be involved in MIP-1α inhibition of CFU-A stem cells, this part of this thesis is focused on isolating genes that are involved in the inhibitory pathway induced by MIP-1α in CFU-A stem cells. To do this a PCR-based technique, subtractive hybridisation was used. In order to perform subtractive hybridisation efficiently an appropriate cellular population has to be isolated, in this case the cellular population is the enriched Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin$^-$ cells. Dunlop *et al* observed that upon stimulation of CFU-A/S stem cells into cycle the addition of 100 ng/ml of MIP-1α was sufficient to induce quiescence after 4 hours (Dunlop *et al* 1992). Other studies within our group indicated that 100 ng/ml of MIP-1α has been observed to produce a consistent inhibition of CFU-A colony formation. Furthermore, Maltman *et al* observed that MIP-1α can induce transcription of the TGF-β gene after 4 hours; therefore the enriched Sca-1^+/ Thy-1.1 $^{-/lo}$/ lin$^-$ cells were incubated for 4 hours with 100 ng/ml MIP-1α or RANTES. Each enrichment of CFU-A stem cells yields approximately 2-5 x 10^4 Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin$^-$ cells, this small number of cells produces an insufficient amount of mRNA for northern blot analysis. Therefore, to overcome the problem of lack of mRNA, the generation of cDNA by oligo dT priming from the two mRNA populations was performed. Messenger RNA (mRNA) was isolated from these cells using an Invitrogen micro-fast track kit, as described in methods section 4.35.1, and cDNA was generated. The cDNA was then amplified via PCR to ensure there was sufficient material to perform the subtraction. The kinetics of PCR means that it will favour amplification of smaller DNA fragments, and thus in an attempt to overcome the size bias the cDNA was cleaved with a restriction enzyme, Alu-1. Alu-1 has a four base recognition site and therefore, will cut the cDNAs approximately every 4^4 (256) base pairs (Old 1989). This has the effect of reducing the size of the DNA molecules to between 200 bp and 1 kb and produces a population of cDNA that is a more representative of the RNA isolated. This particular restriction enzyme is a "flush end" cutter and therefore in order to perform subtractive hybridisation and PCR, specific

double stranded oligonucleotide linkers were ligated onto the cDNA populations (figure 5.10). In an attempt to minimise the possibility of contamination by non-specific driver sequences in the final subtractive product specific restriction sites were engineered into each of the distinct linkers. Single stranded primers from the double stranded linkers were used as PCR primers to allow the generation of large amounts of cDNA and this was used as the starting material for the subtraction using a modified method routinely used by Dr G Brady (Brady and Iscove 1993).

Three rounds of subtraction were performed using cDNA extracted from RANTES treated Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin^- enriched cells as the driver population, and cDNA from MIP-1α treated Sca-1^+ / Thy1.1 $^{lo/-}$ / Lin^- cells as the tracer population. This will produce cDNA fragments that are up regulated in primitive cells exposed to MIP-1α. To confirm the success of the subtractive hybridisation it is normal to examine the cDNA populations for patterns of predictable cDNA alterations. In this case there are no identifiable candidate genes that will be up regulated upon MIP-1α induced inhibition however, it is possible to screen for the removal of housekeeping genes to give an indication that the subtraction has at least removed common genes. As shown in figure 5.11, southern blot analysis examined the abundance of two housekeeping genes β-actin and GAPDH. It can be observed, that on comparing the starting material with each subsequent round of subtraction that the third round of subtraction effectively removes both housekeeping genes. The final cDNA population was amplified by PCR and digested with Not-1. The recognition site for Not-1 had been previously engineered into the tracer specific linkers (figure 5.10). These fragments were then ligated into a pSK Bluescript vector and subsequent transformation of DH5α cells. Blue/white selection was performed on the transformed cells and from the resulting plates, 50 white colonies were picked and grown up in a 5 ml mini-prep culture overnight. A Quiagen mini prep kit was used to purify the plasmid DNA from the overnight cultures and diagnostic digests were performed to check for inserts within the plasmids. Sequence analysis was performed on 50 clones by comparing the sequences produced from the subtractive library with those of known genes and expressed sequence tags (EST's) from non redundant and

Fig 5.10

Pre subtractive hybridisation manipulations

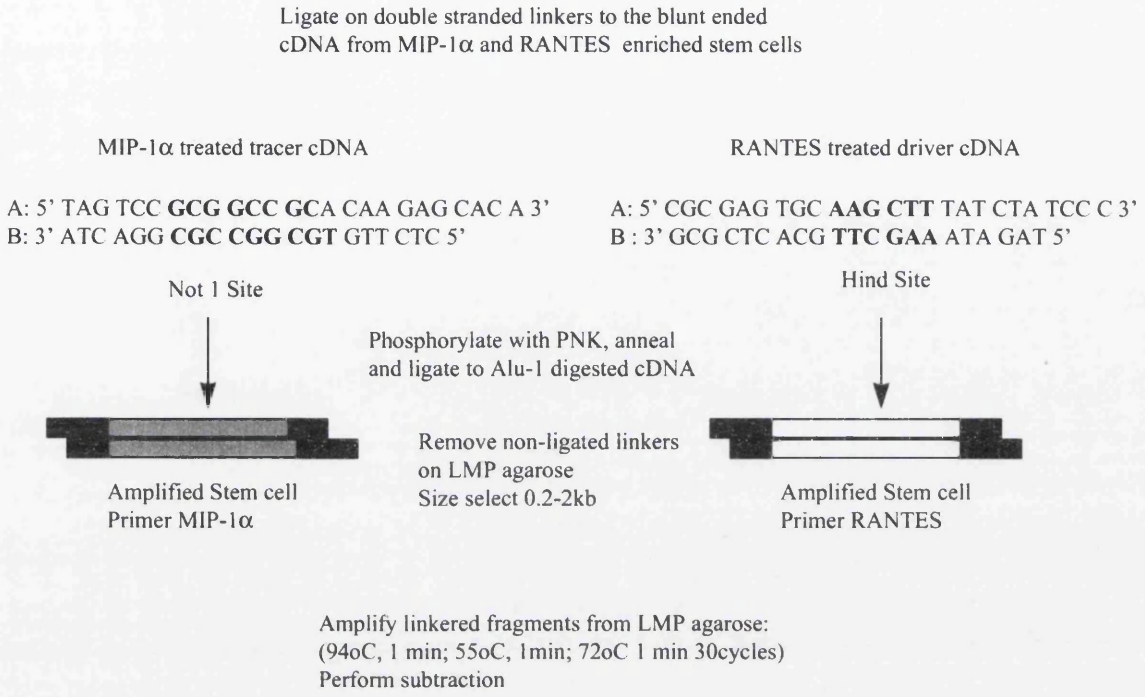


Figure 5.10 Schematic of Manipulations of the cDNA Populations before Subtractive Hybridisation was Performed.

Fig 5.11

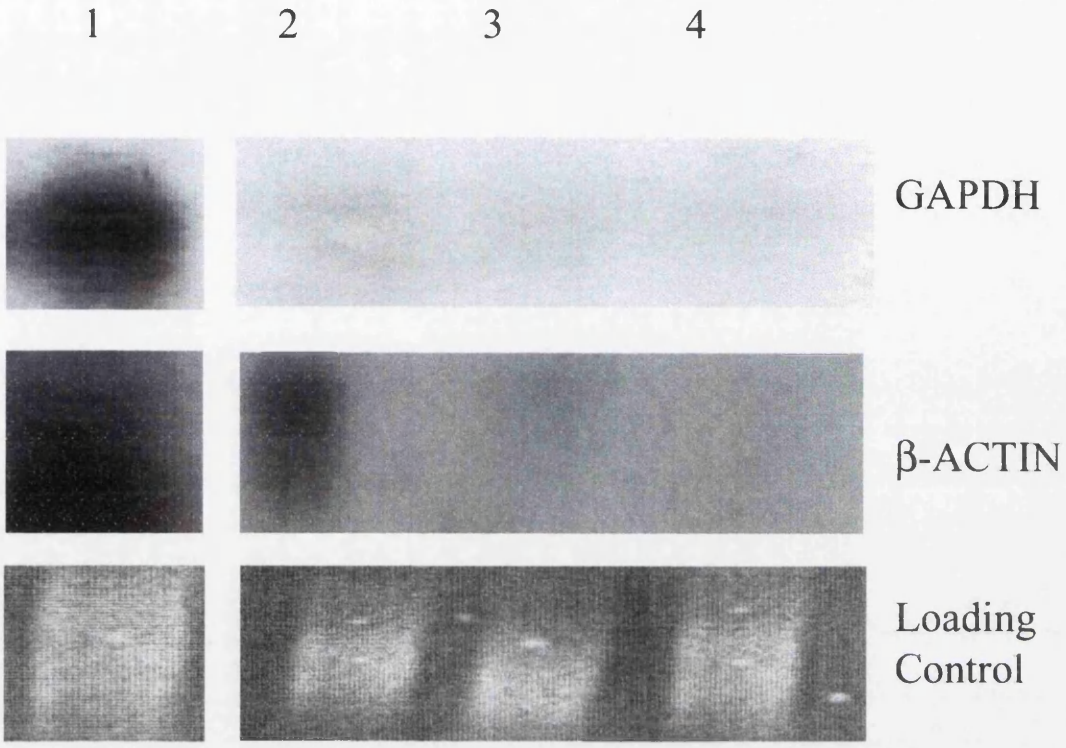


Figure 5.11 Southern blot analysis of GAPDH and β-actin expression on starting cDNA (non subtracted material) and subtracted cDNA

The top row shows a blot hybridised with a probe for GAPDH and the bottom row shows the same blot stripped and reprobed for β-actin. Column 1 represents cDNA from Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells, non subtracted, columns 2, 3 and 4 represent cDNA that has been produced from three subsequent rounds of subtractive hybridisation. Briefly, this involved incubating two populations of Sca-1^{+}/Thy-1.1 ^{lo}/Lin^{-} cells with either 100 ng/ml of MIP-1α or RANTES for 4 hours. mRNA was isolated from these cells and the cDNA that was then produced was used to perform subtractive hybridisation as described in the materials and methods. (Rantes cDNA was used as the driver population and MIP-1α as the tracer population) As there are no known sequences that will be up-regulated in the subtracted material the use of removal of common house keeping genes will give a rough indication that thus far the subtractive hybridisation has at least removed these common genes.

EST databases using the basic logical alignment search tool (BLAST) search program (Altschul *et al* 1990a), (Table 3). Of the 50 clones sequenced there were 20 that did not produce a readable sequence. Although the removal of the common house keeping genes β-actin and GAPDH from the final round of subtraction was encouraging, the analysis of the 30 sequences suggested that the subtractive hybridisation had not worked efficiently. This may be due to the incomplete removal of shared sequences or contamination of the subtracted material with inappropriately amplified driver sequences. Genes that are fundamental for the every day functions of the cell were identified, for example Elongation factor-1δ a gene involved in DNA replication and a human aldehyde reductase (Bohren *et al* 1989). Aldeyhde reductase is one of a number of cytosolic, monomeric oxidoreductases that catalyse the NADPH-dependant reduction of a wide variety of carbonyl compounds such as sugars, glucuronate as well as xenobiotic aldehydes and ketones (Wermuth 1985).

5.9 Summary

The enrichment process isolated a population of cells from bone marrow that were depleted of lineage positive cells and were stained highly for the Sca-1 antigen and low to negative Thy1.1 antigen expression. These Sca-1^{+} / Thy1.1 $^{lo/-}$ / Lin^{-} cells have been observed to produce a cloning efficiency of 22 % in the CFU-A assay and the formation of these CFU-A colonies were almost completely inhibited by MIP-1α. For the reasons explained in section 5.7, the cDNA extracted from this cellular population was used as for the subtractive hybridisation. The removal of the house keeping genes β-actin and GAPDH during the subtraction (figure 5.11) gave an initial indication that the subtraction had worked. However, the results outlined in table 3, suggested that the subtractive hybridisation has not effectively removed sequences that are shared between the driver and tracer populations.

Table 3

Clone Number	Identity	Accession Number	Length (bp)	Journal if published		
1, 2,3 6, 7,11,32, 37,43	Stragene Lung Carcinoma sequence 937218 Homo sapiens cDNA clone	AA 134546	469	Genome Res 6 807-828 1996		
5	Mouse BAC-146N21 Chromosome X contains idurinate-2-sulfatase gene	AC002315	110079			
4,8,9,16, 25,27,36, 41,45	H. sapiens seryl-tRNA Synthetase	X91257	1846	Eur.J. Biochem 250 77-84 1997		
12,40	Homo Sapiens Chromosome 5, P1 clone 1338G6 (LBNL H35) complete sequence	AC004502	79716	Unpublished		
15	Human aldehyde reductase	J04794	1132	J Biol Chem 264 9547-9551 1989		
17,26,39,4 0	Elongation Factor -1delta	NM 001960	991	Biochem Biophys Acta 1174 87-90 1993		
29	Homo sapiens BAC clone GS 250A16 from 7p21-p22, complete sequence	AC005019	188362	Submitted		
31	Zf72a09.r1 Soares pineal gland N3HPG Homo sapiens cDNA clone	AA069743	526	Genome Res 6 807-828 1996		
35	Mus musculus adult C57BL/6J testis Mus musculus cDNA clone	AV047894	289	Unpublished		
42	Rat Carbohydrate binding receptor gene					
44	Homo sapiens chromosome 16, BAC clone RPCI-11_192K18 complete sequence	AC 006075	169765	Unpublished		

Table 4 Analysis of Sequences isolated from the subtractive hybridisation

Sequences produce from the subtractive library were analysed for homologies with known genes and expressed sequence tags (EST's) from non redundant and EST databases using the basic logical alignment search tool (BLAST) search program (Altschul *et al* 1990a, b).

CHAPTER 6

RESULTS 2 : The alteration of SCF and M-CSF concentration on the inhibition of CFU-A colony formation by MIP-1α

6.1 Introduction

Unfortunately, as outlined in the previous chapter, the examination of gene expression induction or repression by MIP-1α in CFU-A stem cells by subtractive hybridisation was unsuccessful. Therefore, it was decided to investigate the interaction of MIP-1α and the CFU-A growth factors upon colony formation. The specific growth factors within the CFU-A assay produce the signals that allow the CFU-A stem cells to survive and thus differentiate into the CFU-A colonies. Upon the addition of MIP-1α to a CFU-A assay it can be observed that the CFU-A colony formation is inhibited, suggesting that the MIP-1α signalling pathway interacts with the other growth factor signalling pathways in some way thus preventing the formation of CFU-A colonies. Therefore, it was decided to investigate the possibility of interfering with the ability of MIP-1α to inhibit the formation of CFU-A colonies by altering the concentration of each of the individual growth factors.

6.2 The Effects of Conditioned Media and Recombinant Growth Factors on CFU-A Colony Formation

With the use of conditioned media (CM) it is impossible to be certain what growth factors are being added to the assay system therefore, the replacement of CM with recombinant growth factors will rule out this uncertainty. Indeed, Pragnell and colleagues previously demonstrated that recombinant human M-CSF (6 ng/ml), recombinant murine GM-CSF (0.2 ng/ml) and recombinant murine SCF (12 ng/ml) can be used instead of CM for effective CFU-A assay development (Pragnell *et al* 1994). However, before any alteration of recombinant growth factor concentrations were performed, it was deemed necessary to confirm that there was no difference in CFU-A colony production in CFU-A assays containing conditioned media or the above concentrations of recombinant growth factors. In the first experiment, 5000 bone marrow cells per plate were added to a CFU-A assay containing 10 % L929 CM

Fig 6.1

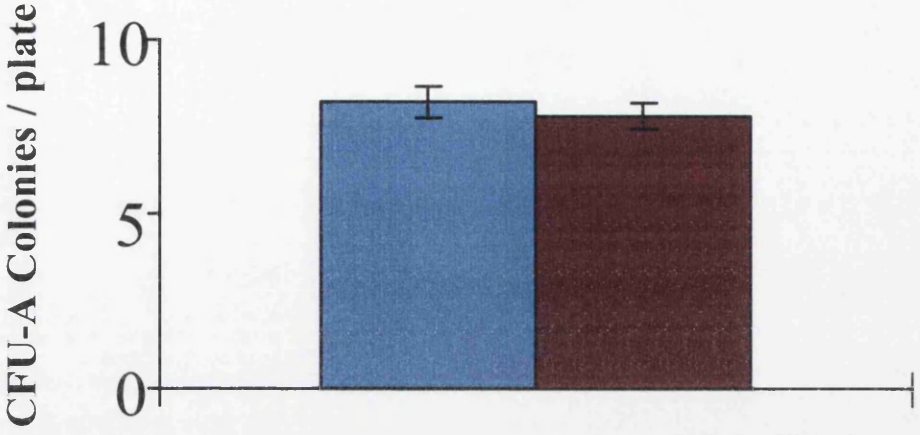


Figure 6.1 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony production by using conditioned media or recombinant growth factors

A CFU-A assay was set up containing either rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and rm SCF at 12 ng/ml or 10% L 929 and 10 % AF-1.19T conditioned media. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Conditioned media (CM)
- Recombinant growth factors

and 10 % AF1 19T CM or 0.2 ng/ml rm GM-CSF, 6 ng/ml rh M-CSF and 12 ng/ml rm SCF as the source of growth factors. From figure 6.1, it can be observed that there is no significant difference between the CFU-A colony formation in either growth condition. Both conditioned media and recombinant growth factors produce approximately 8 CFU-A colonies per 5000 bone marrow cells. This figure is in agreement with previous work that has shown that the incidence of CFU-A in normal bone marrow is $150\text{-}220/10^5$ (Lorimore *et al* 1990).

Experiments were then set up to examine if altering the source of growth factors had any effect on the ability of MIP-1α to inhibit CFU-A colonies. MIP-1α was added to the plates at 0.5, 1, 5, 10, 50 and 100 ng/ml and only 100 and 50 ng/ml of MIP-1α produced a statistically significant inhibition of CFU-A colony formation under both growth conditions ($P < 0.001$) (Figure 6.2). The inhibition of CFU-A colony formation at these concentrations is in agreement with previous results within our group that indicated that MIP-1α at 100 or 50 ng/ml reproducibly inhibit the formation of CFU-A colonies. Upon a further 7 days incubation the CFU-A colonies that were inhibited and had a diameter less than 2 mm (approx 0.2- 0.5 mm) were observed to grow and reach the characteristic CFU-A colony size, indicating that the reduction of CFU-A colonies was reversible. Furthermore, it can also be observed that no further inhibition occurs at MIP-1α concentrations lower than 50 ng/ml (figure 6.2). Therefore, these results indicate that the CFU-A assays containing CM or rGFs are functionally indistinguishable.

6.3 Tyrosine Kinase Signalling Growth Factors

SCF has a number of functions in the haemopoietic system. It has been reported to be a survival factor and on its own SCF has no proliferative effect on stem cells however, in combination with other growth factors it mediates extensive proliferation and expansion in cell numbers (Varas *et al* 1997). It plays an important role in megakaryopoiesis and erythropoiesis and it also appears to regulate the production, maintenance and differentiation of mast cells. Unlike SCF, macrophage colony stimulating factor (M-CSF) is a lineage specific growth factor which has been reported to play an essential role in the survival, proliferation, differentiation, and the maturation of the mononuclear phagocytic lineage (Fixe *et al* 1997). M-CSF and SCF are two of the three growth factors that are essential for the differentiation of CFU-A

Fig 6.2

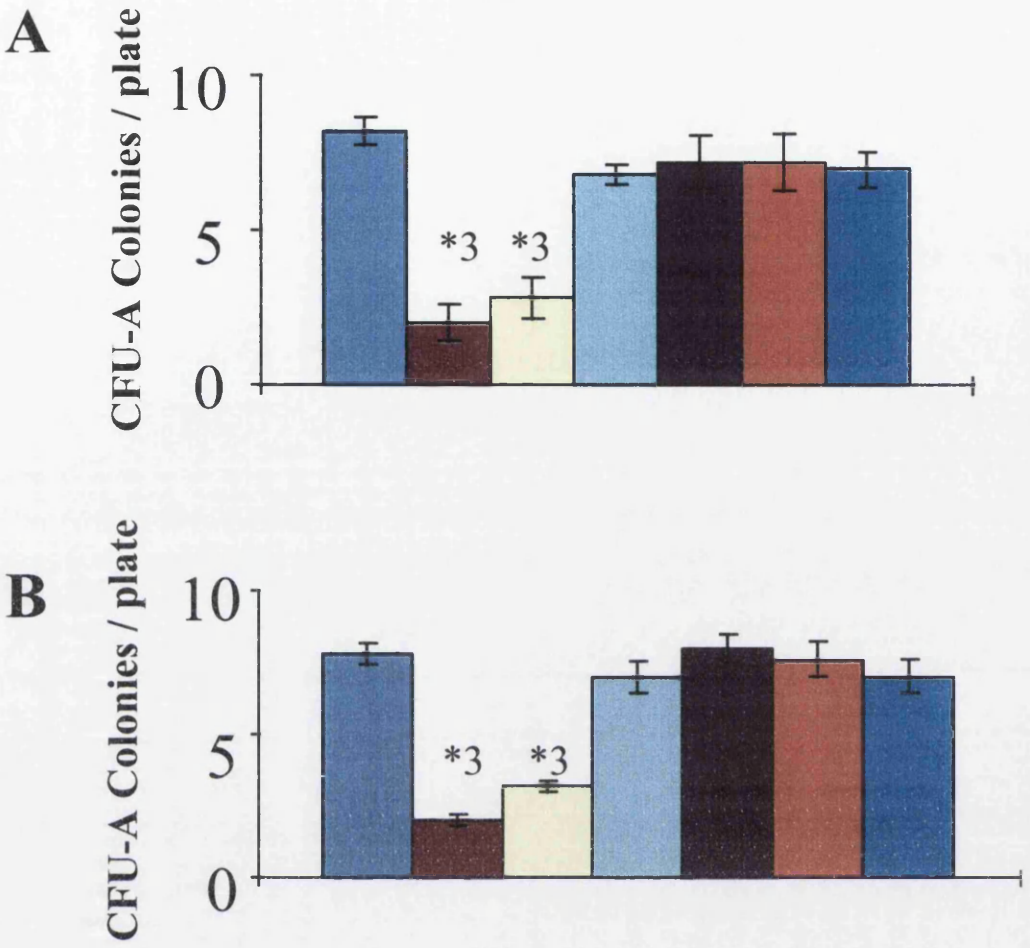


Figure 6.2 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production by using conditioned media or recombinant growth factors

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml were added to the CFU-A assay containing either rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and rm SCF at 12 ng/ml or 10 % L 929 and 10% AF-1.19T conditioned media. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$). Figure 6.2A contains the results from a CFU-A assay grown in conditioned media (10 % L 929 and 10 % AF-1.19T). Figure 6.2B contains the results from a CFU-A assay grown in recombinant growth factors (rm GM-CSF 0.2 ng/ml, rh M-CSF 6 ng/ml and 12 ng/ml of rm SCF).

- CM or rGF
- CM or rGF + 100 ng/ml MIP-1α
- CM or rGF + 50 ng/ml MIP-1α
- CM or rGF + 10 ng/ml MIP-1α
- CM or rGF + 5 ng/ml MIP-1
- CM or rGF + 1 ng/ml MIP-1α
- CM or rGF + 0.5 ng/ml MIP-1α

stem cells into CFU-A colonies and they produce their various activities through the interaction with their specific receptors, *c-fms* and *c-kit* which are members of the type III receptor tyrosine kinase family. This family of cytokine receptors also includes flt3 and both of the receptors for platelet derived growth factor, PDGFR A and B. Each receptor is approximately 1000 amino acids in length, has five Ig-like domains in the extra cellular region, and contains a split catalytic domain in the cytoplasmic region that phosphorylates tyrosine residues in specific target proteins after activation of the receptor by its ligand (Sherr 1990). In this part of the project the concentration of SCF and M-CSF were individually altered in an attempt to examine the ability of the tyrosine kinase signalling pathway to interfere with the MIP-1α inhibitory pathway.

6.3.1 The Effects of SCF and M-CSF on CFU-A Colony Formation

The role of the tyrosine kinase signalling pathway and its ability to interact or interfere with the inhibition of CFU-A colony formation was examined by altering the concentration of rm SCF or rh M-CSF in the CFU-A assay, in the presence or absence of MIP-1α. In the first experiment, the effect of the alteration of the rm SCF concentration was examined. Recombinant murine GM-CSF and rh M-CSF were added to the CFU-A assay at normal levels, 0.2 and 6 ng/ml, and SCF was either omitted or added at 0.0012, 0.012, 0.12, 1.2, 12 and 120 ng/ml (figure 6.3). The omission of rm SCF from the assay had the effect of significantly reducing the colony number from an average of 9.2 to an average of 2.8 CFU-A colonies ($p < 0.001$). Although these colonies have been scored as CFU-A colonies, it is possible that they are not CFU-A colonies and have arisen from the increased growth of committed progenitors such as CFU-GM. Granulocyte/ macrophage colony forming unit (CFU-GM) colonies are normally grown in agar assays containing between 0.1-10 ng/ml of GM-CSF, their incidence within the bone marrow has been previously reported to be between 100-240 / $1x10^5$ bone marrow cells (Broxmeyer *et al* 1991b). As the concentrations of rm GM-CSF used in the above experiment are at the lower range of the scale observed by Broxmeyer for the formation of these CFU-GM colonies this may explain the slight reduction in the observed number of CFU-GM colonies. However, it is also likely that the omission of rm SCF from the assay in figure 6.3 would mean that within the assay there would not be sufficient growth factor activity to induce CFU-A stem cells to develop into their characteristic colony size.

Fig 6.3

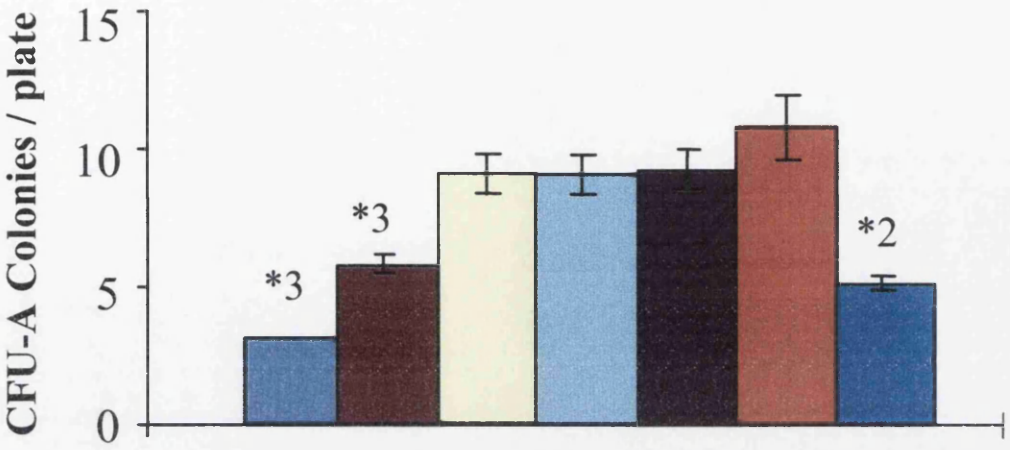


Figure 6.3 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony production by increasing or reducing the SCF concentration within the assay

A CFU-A assay was set up with normal levels of rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and rm SCF at 0, 0.0012, 0.012, 0.12,1.2, 12 and 120 ng/ml. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing normal SCF levels (12 ng/ml) to all the other concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- 0 SCF
- 0.0012 ng/ml SCF
- 0.012 ng/ml SCF
- 0.12 ng/ml SCF
- 1.2 ng/ml SCF
- 12 ng/ml SCF
- 120 ng/ml SCF

Therefore, further investigation into the production of these colonies is needed to identify these colonies and ascertain their clonal cell of origin. Upon the addition of 1.2 pg/ml of SCF it can be observed in figure 6.3, that rm SCF can act synergistically with the other growth factors to significantly increase the colony number from an average of 2.8 to an average of 5.6 CFU-A colonies. The colony number is increased further to approximately 8.0 upon the addition of 0.012, 0.12 and 1.2 ng/ml of in SCF and a further increase in colony number to 9.2 is observed on the addition of 12 ng/ml of SCF. The further increment in concentration of SCF to 120 ng/ml had a surprising effect in that it reduced the colony size significantly and thus decreased the average CFU-A colony number to 4.6 ($p < 0.01$).

A similar experiment was set up to examine if the alteration in M-CSF concentration had a similar effect as the alteration of SCF concentration. In this experiment, rm GM-CSF and rm SCF are at normal levels whereas rh M-CSF was omitted or added at 0.0006, 0.006, 0.06, 0.6, 6 and 60 ng/ml to the assay. The removal of M-CSF from the assay reduced the CFU-A colony number from 10.3 to 2.4 colonies ($p < 0.001$), as outlined in figure 6.4. This limited colony formation concurs with a previous study by Pragnell *et al*, they observed that M-CSF is essential for CFU-A colony formation (Pragnell *et al* 1988). The increase in M-CSF concentration in 10 fold increments from 0.6 pg/ml to 6 ng/ml produced an increase in CFU-A colony production in a dose dependant manner culminating in an average of 10.3 colonies being observed at 6 ng/ml, the normal concentration of M-CSF. Upon a further increment in M-CSF concentration to, 60 ng/ml, a similar reduction in CFU-A colony formation to that observed at high doses of stem cell factor was observed to produce a decrease from 10.4 to 6.5 ($p < 0.001$) as shown in figure 6.4. These results indicate that a 10-fold increase in either M-CSF or SCF concentration produces a reduction in the number of CFU-A colonies compared to control assays.

This reduction in CFU-A colony formation induced by high levels of M-CSF or SCF was reversible and appears similar to the reversible inhibition produced by MIP-1α. Furthermore, this reduction is not due to a toxic effect of these growth factors as CFU-A colonies can be observed to grow in these high growth factor concentrations. M-CSF and SCF receptors are members of the tyrosine kinase receptor family therefore; the observed reduction in colony formation may be due to the activation of the same tyrosine kinase-signalling pathway by these separate growth factors.

Fig 6.4

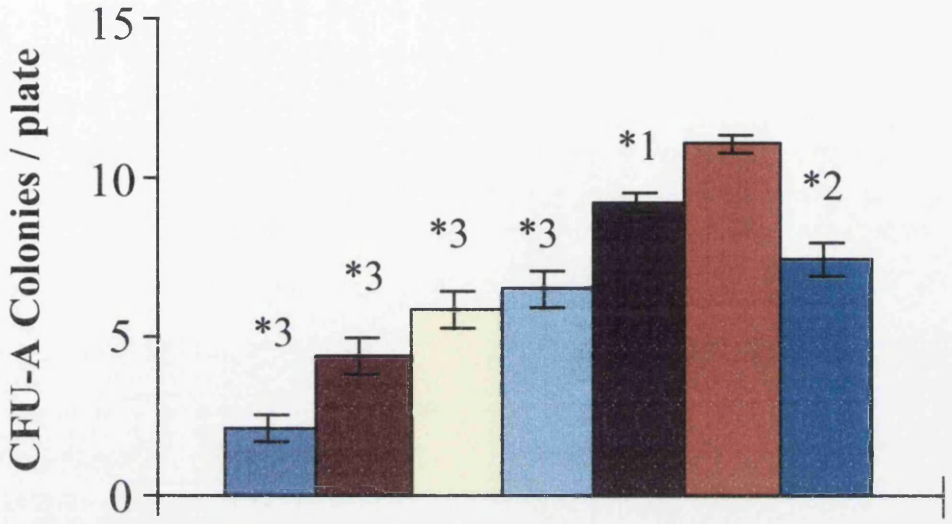


Figure 6.4 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony production by increasing or reducing the rh M-CSF concentration within the assay

A CFU-A assay was set up with normal levels of rm GM-CSF (0.2 ng/ml), rm SCF (12 ng/ml) and rh M-CSF at 0, 0.0006, 0.006, 0.06, 0.6, 6 and 60 ng/ml. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing normal M-CSF levels (6 ng/ml) to all the other concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- 0 M-CSF
- 0.0006 ng/ml M-CSF
- 0.006 ng/ml M-CSF
- 0.06 ng/ml M-CSF
- 0.6 ng/ml M-CSF
- 6 ng/ml M-CSF
- 60 ng/ml M-CSF

6.3.2 The Effects of SCF and M-CSF on the Constituent Cells of the CFU-A Colonies

The effect of altering the SCF and M-CSF concentration on the production of the constituent cell types and their abundance within the CFU-A colonies was next examined (tables 4 and 5). This was performed by mixing unfractionated bone marrow cells with methylcellulose (#3230, -CSF-1 and -EPO), and layering this on top of a standard lower agar layer (section 4.15). Colonies were allowed to form for 11 days at 37 °C in a humidified environment containing 10 % O_2, 5 % CO_2 and 85 % N_2 and then normal and inhibited colonies were picked, washed and counted (Table 4). It was observed that normal CFU-A colonies predominantly consist of macrophages, approximately 98 %, with a small number of granulocytes, normally neutrophils, and blast cells. The data presented in table 4 and 5 concurs with the findings of Pragnell *et al*, who previously reported that CFU-A colonies derived from mouse bone marrow are composed mainly of macrophages (90-95%). Furthermore, it also demonstrates that altering the rm SCF concentrations within the CFU-A assay has little effect on the constituent cells of the CFU-A colonies. On the addition of 100 ng/ml of MIP-1α to a normal CFU-A it can be observed that there is a non-significant reduction in the percentage of macrophages and a non-significant increase in the percentage of granulocytes in the inhibited CFU-A colonies. The data in table 4, also indicates that the addition of MIP-1α to a CFU-A assays containing normal levels of each growth factor reduces the number of cells/colony from 21800 to 7000 cells. Interestingly in the CFU-A assays containing 120 ng/ml rm SCF, the number of cells/ colony is reduced from 21800 with 12 ng/ml to 16600 with 120 ng/ml and, upon the further addition of 100 ng/ml of MIP-1α the number is further reduced to 4900 cells. Although the alteration in SCF concentration does not effect the proportions of the constituent cells of the CFU-A colonies it does as suggested by the results in table 4 reduce the number of cells/colony.

A similar experiment was set up to examine the effects on the number and constituent cells of the CFU-A colonies in the alteration of rh M-CSF in the presence or absence of 100 ng/ml of MIP-1α. From the data in table 5, it can be observed that there is a

Table 4

Cell Numbers within CFU-A colonies

rm SCF 1.2 ng/ml	20400
rm SCF 1.2 ng/ml + MIP-1α	5800
rm SCF 12 ng/ml	21800
rm SCF 12 ng/ml + MIP-1α	7000
rm SCF 120 ng/ml	16600
rm SCF 120 ng/ml + MIP-1α	4900

Differential counts (500 cells)

	Macrophages	Granulocytes	Others
rm SCF 1.2 ng/ml	495	3	2
rm SCF 1.2 ng/ml + MIP-1α	496	3	1
rm SCF 12 ng/ml	495	4	1
rm SCF 12 ng/ml + MIP-1α	492	8	0
rm SCF 120 ng/ml	492	4	4
rm SCF 120 ng/ml + MIP-1α	494	2	4

Table 4 Analysis of cell number and morphology of the cells within the CFU-A colonies containing 1.2, 12 and 120 ng/ml of rm SCF in the presence or absence of 100 ng/ml MIP-1α

CFU-A assays were set up containing a top layer of methylcellulose instead of the normal double agar assay. Three sets of 10 colonies were picked from plates containing 100 ng/ml MIP-1α and three sets of 5 colonies from the plates that did not contain MIP-1α. These cells were pooled, washed and either cytospins were prepared and stained with Giemsa for morphology analysis or cell numbers were counted.

Table 5

Cell Numbers within CFU-A colonies

rh M-CSF 0.6 ng/ml	24600
rh M-CSF 0.6 ng/ml + MIP-1α	9600
rh M-CSF 6 ng/ml	22850
rh M-CSF 6 ng/ml + MIP-1α	11200
rh M-CSF 60 ng/ml	20600
rh M-CSF 60 ng/ml + MIP-1α	8500

Differential counts (500 cells)

	Macrophages	Granulocytes	Others
rh M-CSF 0.6 ng/ml	495	3	2
rh M-CSF 0.6 ng/ml + MIP-1α	490	8	2
rh M-CSF 6 ng/ml	490	5	5
rh M-CSF 6 ng/ml + MIP-1α	495	5	0
rh M-CSF 60 ng/ml	485	10	5
rh M-CSF 60 ng/ml + MIP-1α	479	15	6

Table 5 Analysis of cell number and morphology of the cells within the CFU-A colonies containing 0.6, 6 and 60 ng/ml of rh M-CSF in the presence or absence of 100 ng/ml MIP-1α

CFU-A assays were set up containing a top layer of methylcellulose instead of the normal double agar assay. 10 colonies were picked from plates containing 100 ng/ml MIP-1α and 5 colonies from the plates that did not contain MIP-1α. These cells were pooled, washed and either cytospins were prepared and stained by Giemsa for morphology analysis or cell numbers were counted.

non-significant reduction in the number of cells within the colonies from 22850 with 6 ng/ml to 20600 with 60 ng/ml. However, data from table 5 indicates that there is a reduction in the number of cells within the CFU-A colonies in assays containing 60 ng/ml of rhM-CSF compared to assays containing 0.6 ng/ml of rhM-CSF. This therefore suggests that the alteration in rhM-CSF concentration is effecting the numbers of cells in each CFU-A colony. On the further addition of MIP-1α there is a significant reduction in cell number to 11200 in normal assay conditions and 8500 cells in assays containing a 10-fold increase in M-CSF concentration. These results indicate that although the increase in M-CSF reduces the overall cell numbers within the CFU-A colonies this reduction is not significant and, only upon the inclusion of MIP-1α does this reduction in cell number become significant. On examining the composition of the CFU-A colonies it can be observed that 0.6 ng/ml rh M-CSF +/- 100 ng/ml MIP-1α does not alter the constituent cells of the CFU-A colony significantly from assays containing 6 ng/ml of rh M-CSF +/- 100 ng/ml MIP-1α. However, on the addition of 60 ng/ml rh M-CSF +/- 100 ng/ml MIP-1α it was observed that there is a significant increase in the number of granulocytes with a concurrent reduction in the number of macrophages. The observed decrease in CFU-A colonies at high levels of SCF and M-CSF was hypothesised to be due to the expression of an inhibitory factor such as MIP-1α or alternatively, due to the induction of self renewal of the CFU-A stem cells at the expense of differentiation. A report by Baghestanian *et al* observed that SCF in combination with PMA can induce de novo expression of MIP-1β mRNA and MIP-1α mRNA in a mast cell line (HMC-1) (Baghestanian *et al* 1997). Like SCF, M-CSF has also been reported to induce the expression of MIP-1α and interestingly, Jarmin *et al* also observed that MIP-1α and MIP-1β mRNA expression and a corresponding release of the MIP-1α and MIP-1 β proteins can be induced by GM-CSF in bone marrow macrophages (Maltman *et al* 1996, Jarmin *et al* 1999). Furthermore, a more recent study has indicated that haemopoietic stem cells can also express MIP-1α (Majka *et al* 1999). Therefore, if as proposed, high levels of M-CSF or SCF alone or in conjunction with GM-CSF may induce the expression of MIP-1α within the constituent cells of the CFU-A colonies. If this were the case it might be expected that concentrations of MIP-1α lower than previously observed within our group would induce inhibition of CFU-A colony formation.

6.3.3 The Effects of SCF and M-CSF on MIP-1α Inhibition of CFU-A Colony Formation

To examine if this observed reduction in CFU-A colony size was due to the induced expression of MIP-1α by high levels of M-CSF or SCF, a CFU-A assay was set up to examine the effects of varying concentrations of rm SCF or rh M-CSF on a dose response of MIP-1α inhibition. In figure 6.5, MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to CFU-A assays containing normal levels of rm GM-CSF and rh M-CSF with rm SCF at 0.0012, 0.012 and 0.12, 1.2, 12, 120 ng/ml. MIP-1α at 100 and 50 ng/ml are the only concentrations that were observed to produce a statistically significant inhibition of CFU-A colony production at all of the SCF concentrations in figure 6.5 ($p < 0.001$). If as hypothesised that high levels of rm SCF are producing the reduction of CFU-A colonies by the induction of MIP-1α, it would be expected that this increase in MIP-1α expression in combination with the exogenously added MIP-1α would produce an additive reduction in CFU-A colony formation. However, this does not appear to happen, indeed, quite the opposite occurs as high rm SCF concentration and non inhibitory concentrations of MIP-1α increase the CFU-A colony formation over and above that of the control numbers, $p < 0.05$ and 0.01 (figure 6.5f). Although MIP-1α is predominantly known as an inhibitory molecule it has been observed to produce progenitor cell stimulatory effects in CFU-GM progenitor assays but not so far on CFU-A stem cells. Therefore, it is possible that at these lower concentrations of MIP-1α in conjunction with high levels of rm SCF, that MIP-1α is unable to act as a negative factor but somehow can act as a positive growth factor possibly through an alternative receptor who's expression is regulated by SCF.

A similar experiment was set up to examine if the reduction in CFU-A colony formation in assays containing increased levels of rh M-CSF was through the induction of MIP-1α expression. In figure 6.6 the effect of MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml can be observed, in assays containing rh M-CSF concentrations of 0.0006, 0.006, 0.06, 0.6, 6 and 60 ng/ml. MIP-1α significantly inhibits CFU-A colony formation at 100 and 50 ng/ml in all of the rh M-CSF concentrations, $p < 0.001$.

Fig 6.5

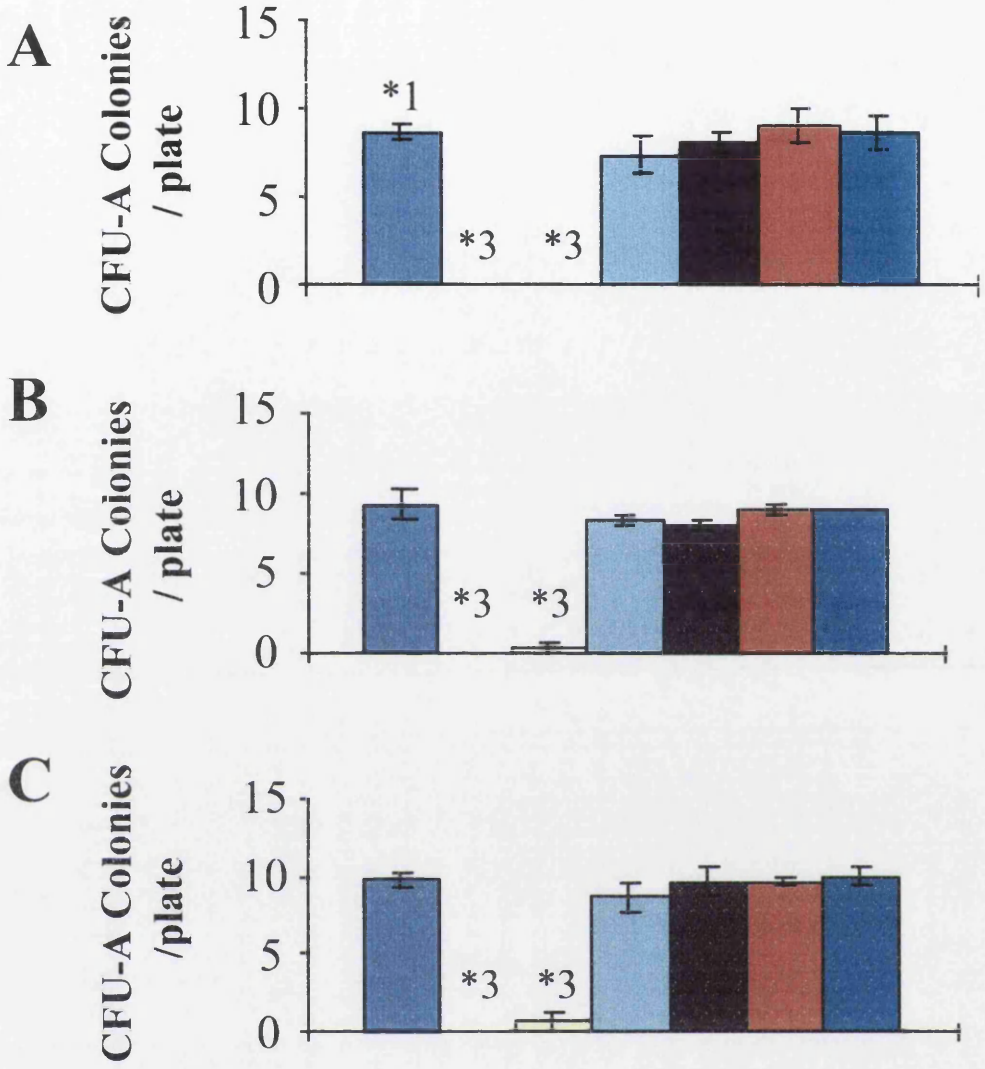


Figure 6.5 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 0.0012, 0.012, 0.12 ng/ml of rm SCF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and either 0.0012, 0.012, 0.12 ng/ml of rm SCF and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (No MIP-1α) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$). Furthermore Students T-test were performed comparing each control to the 12ng/ml contol.

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

Fig 6.5

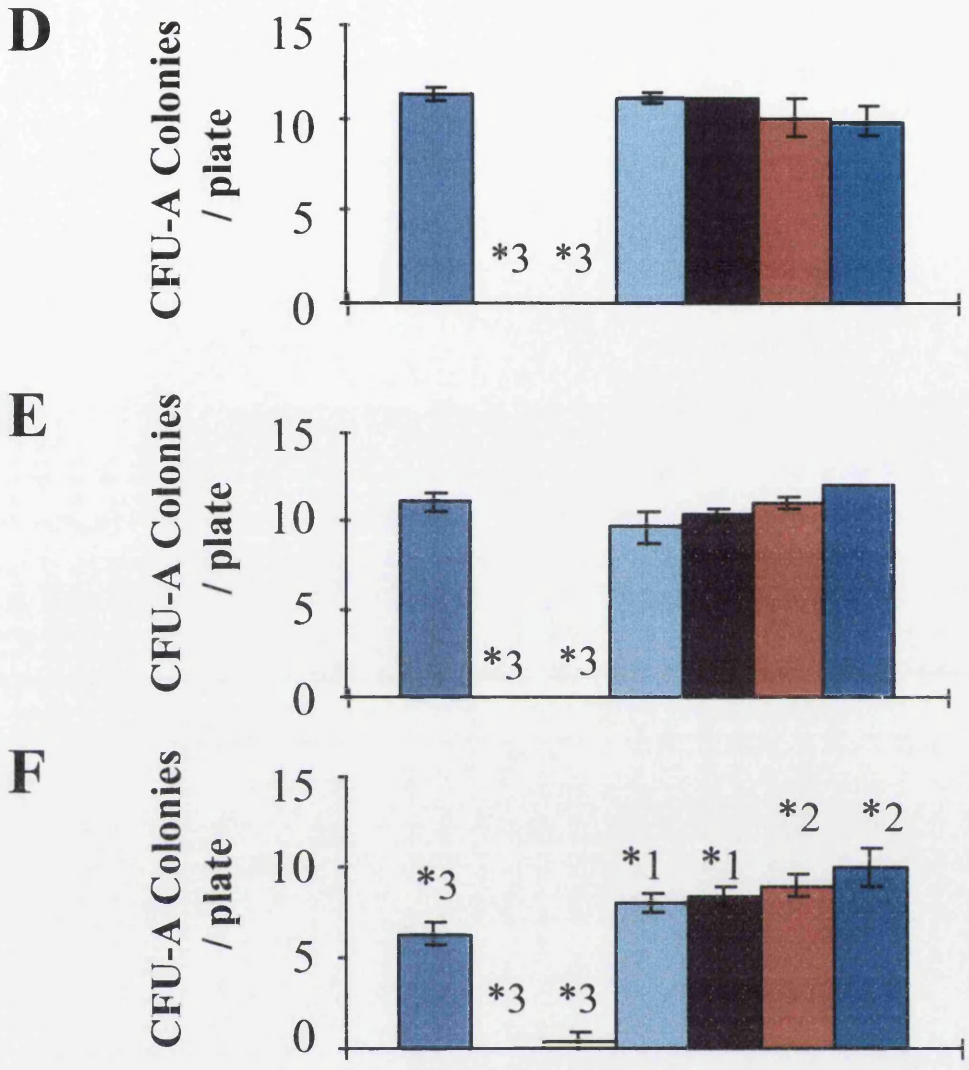


Figure 6.5 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 1.2, 12 and 120 ng/ml of rm SCF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and either 1.2, 12 or 120 ng/ml of rm SCF and these are represented in D, E and F respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (No MIP-1α) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

In figure 6.6C, it can be observed that MIP-1α at 10, 5 and 1 ng/ml reduces the colony formation but this is not statistically significant. When rh M-CSF is added at 6 ng/ml (normal levels) in (figure 6.6E) it can be seen that only 100 and 50 ng/ml MIP-1α inhibit CFU-A colony formation ($p < 0.001$). On the further increment of M-CSF concentration to 60 ng/ml there is a reduction in CFU-A colonies from 11.4 to 7.0. If high levels of rh M-CSF were indeed inducing MIP-1α expression within the CFU-A colonies, it would be expected that MIP-1α concentrations less than 100 and 50 ng/ml would in combination with rh M-CSF induce MIP-1α expression inhibit CFU-A colony formation. However, inhibition only occurs at 100 and 50 ng/ml of MIP-1α ($p < 0.001$) and this suggests that rh M-CSF may not be inducing the expression of MIP-1α. As was the case with SCF the hypothesised reduction in CFU-A colony numbers at concentrations of MIP-1α not normally associated with inhibition in assays containing high levels of M-CSF did not occur. In fact similar to the observed activity with high SCF, non-inhibitory concentrations of MIP-1α increased the formation of CFU-A colonies over and above that of the control numbers, $p < 0.05$ and 0.01, see discussion. As mentioned above MIP-1α has been previously reported to act as a growth stimulator for granulocyte macrophage progenitor cells and a similar stimulatory activity for MIP-1α was also reported by Broxmeyer *et al.* They observed that MIP-1α could enhance the colony formation of a subset of myeloid progenitor cells, CFU-M colony forming unit macrophage, in the presence of 0.6 ng/ml M-CSF (Broxmeyer *et al* 1999). As previously discussed, it is the various growth factors within each assay that induce the formation of the specific colonies via the survival, proliferation and differentiation of their clonal stem/progenitor cells. Therefore, it may be possible that by altering the concentrations of the CFU-A growth factors that the growth conditions may favour the formation of maturer progenitor cell colonies. Alternatively high levels of SCF or M-CSF may alone or in combination with GM-CSF increase the levels of MIP-1α receptor and upon the interaction with MIP-1α this may lead to the increase formation of CFU-A colonies, see discussion.

Fig 6.6

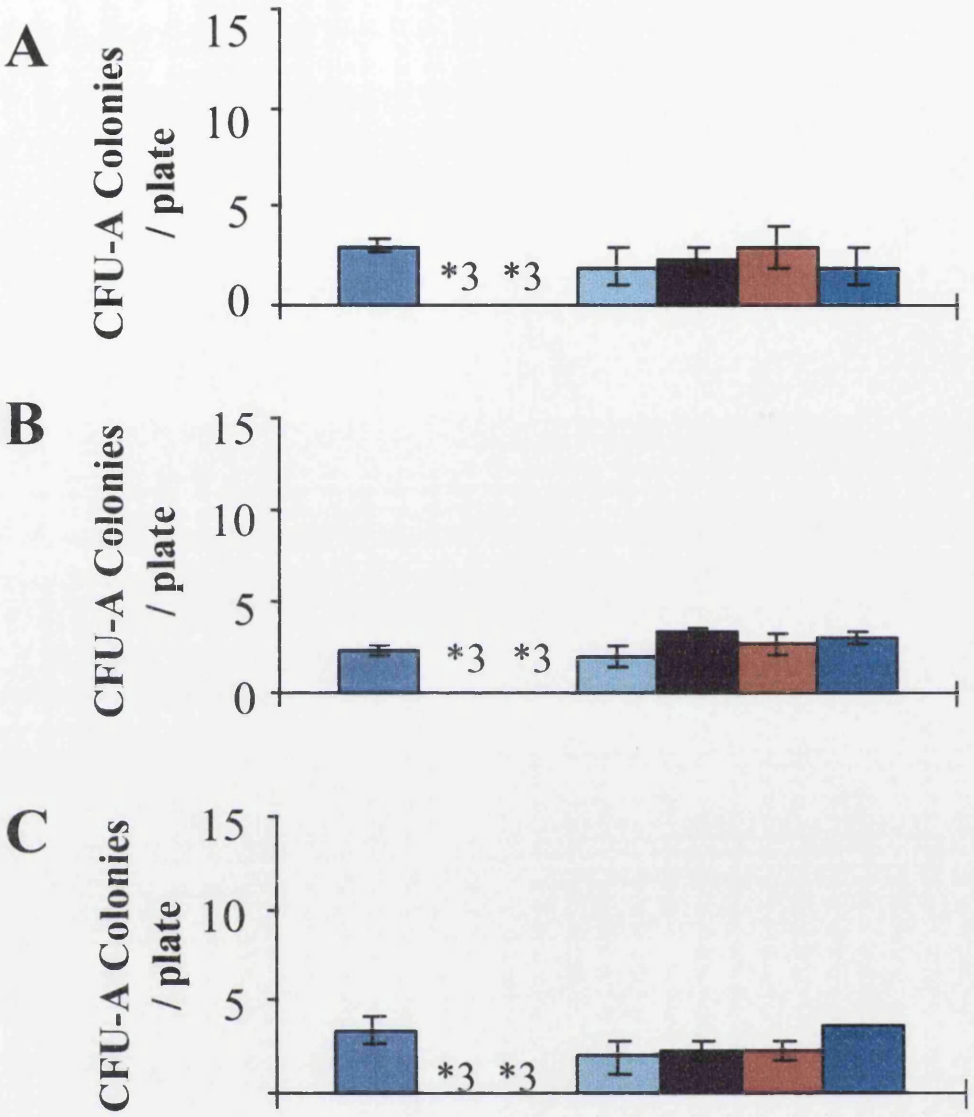


Figure 6.6 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 0.0006, 0.006 and 0.06 ng/ml of rh M-CSF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rm GM-CSF (0.2 ng/ml), (12 ng/ml) rm SCF and rh M-CSF at 0.0006, 0.006, 0.06 ng/ml and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (No MIP-1α) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)..

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

Fig 6.6

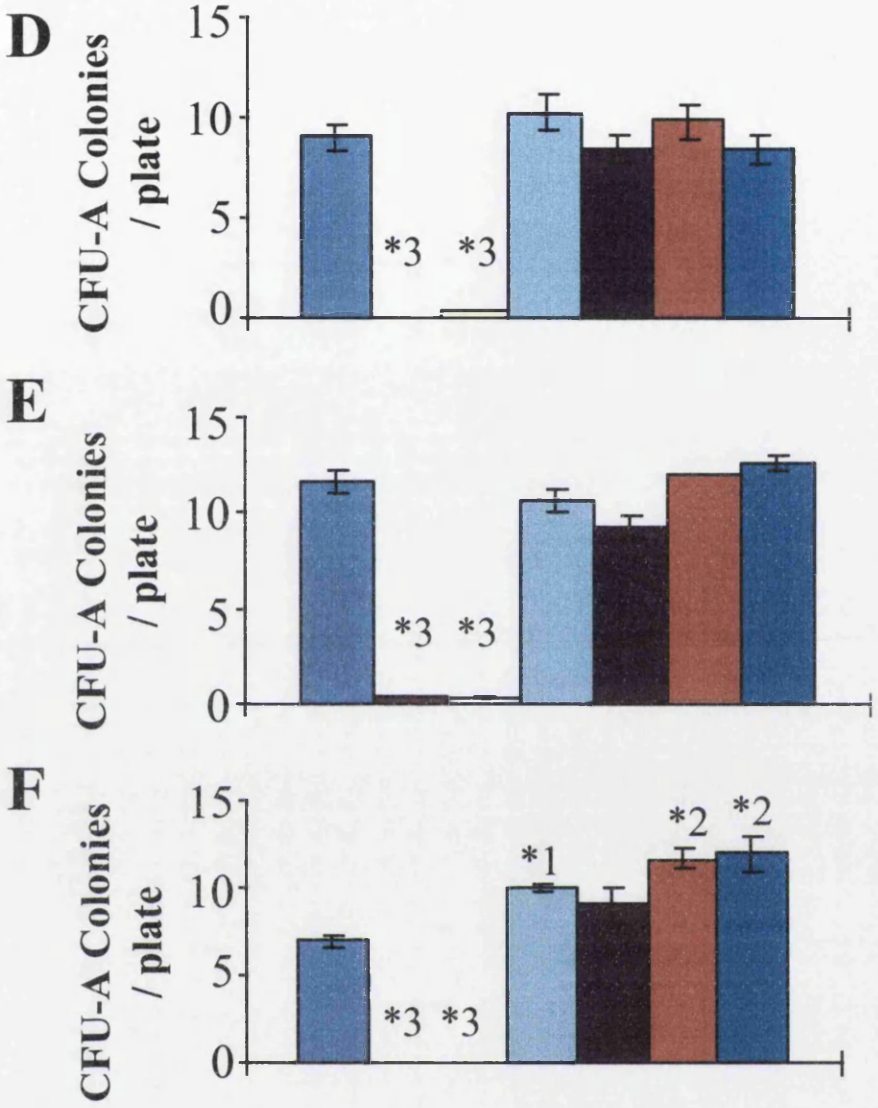


Figure 6.6 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 0.6, 6 and 60 ng/ml of rh M-CSF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rm GM-CSF (0.2ng/ml), (12 ng/ml) of rm SCF and rh M-CSF at 0.6, 6 and 60 ng/ml and these are represented in D, E and F respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (No MIP-1α) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

6.3.4 The Effects of a Neutralising MIP-1α Antibody on CFU-A Colony Formation

To further assess the possibility that the cells within the CFU-A colony could be producing MIP-1α in response to high levels of SCF or M-CSF the effect of the addition of a MIP-1α neutralising antibody to CFU-A assays was examined. A CFU-A assay was therefore, set up containing normal levels of rm GM-CSF, rh M-CSF, rm SCF at 1.2, 12, 120 ng/ml in the presence or absence of 100 ng/ml of MIP-1α and 5μg/ml of the MIP-1α neutralising antibody (Figure 6.7). At control levels of SCF (12 ng/ml) and 1.2 ng/ml an average of 9.8 and 10.3 colonies were produced. In the assays containing 120 ng/ml of SCF a slight reduction in colony number from 9.8 to 8.6 was observed however, this is not significant when compered to control levels. If as previously suggested, that high levels of SCF can induce MIP-1α expression the addition of a MIP-1α neutralising antibody should lead to an increase in colony number, see figure 6.7C. Although there is a slight increase in colony number this is not significant. MIP-1α at 100 ng/ml reduced colony number significantly to 1.0, 1.3, or 0 ($p < 0.001$) in assays containing 1.2, 12 and 120 ng/ml of rm SCF. On the addition of the neutralising antibody to the assay containing 12 and 1.2 ng/ml of SCF the inhibition of CFU-A colony formation was reversed. However, the neutralising antibody did not return the CFU-A colony number back to the control levels in the assay containing 100 ng/ml of MIP-1α and 120 ng/ml SCF ($p < 0.05$). From these results it seems likely that high levels of SCF do not induce the expression of MIP-1α however, as the antibody used above is a MIP-1α neutralising antibody this does not rule out the possible induction of other inhibitory molecules by high levels of SCF.

A similar experiment using the same MIP-1α neutralising antibody was set up to examine whether M-CSF might induce MIP-1α expression in the cells of the CFU-A colony. Figure 6.8, shows a CFU-A assay containing normal levels of rm GM-CSF and rm SCF with the addition 0.6, 6 and 60 ng/ml of rh M-CSF. As well as these growth factors 100 ng/ml of MIP-1α and 5 μg/ml anti MIP-1α antibody were added

Fig 6.7

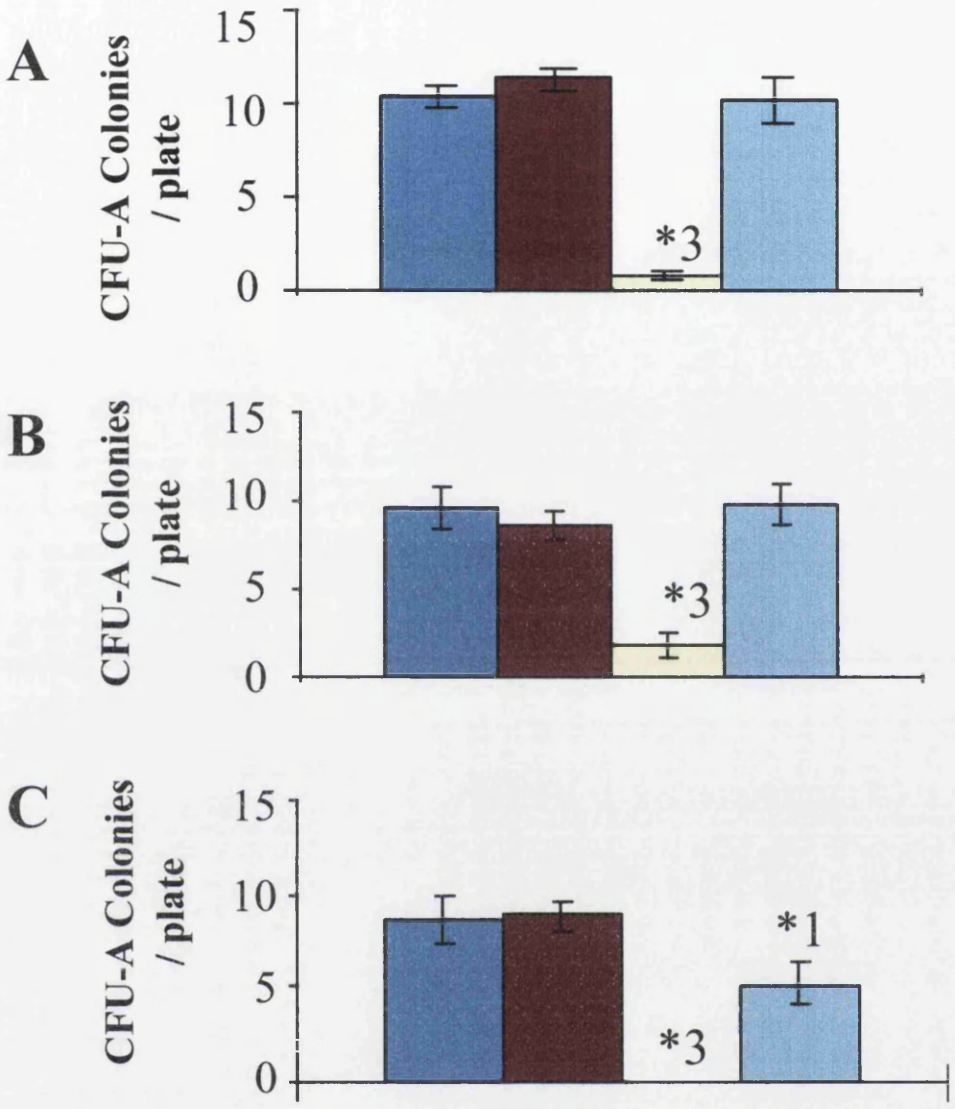


Figure 6.7 Colony Forming Unit Agar (CFU-A) analysis of the effect of anti -MIP-1α antibodies on the size of the CFU-A colonies

Unfractionated bone marrow was plated in a CFU-A assay containing 1.2, 12 and 120 ng/ml rm SCF and these are represented in A, B and C respectively. The effects of MIP-1α at 100 ng/ml and of an anti-MIP-1α antibody at 5 μg/ml on the size and number of the colonies were examined. The average colony production (+ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (normal bone marrow) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Normal bone marrow
- Normal bone marrow 5 μg/ml of anti -MIP-1α antibody
- Normal bone marrow and 100 ng/ml of MIP-1α
- Normal bone marrow 100 ng/ml of MIP-1α plus 5 μg/ml of anti -MIP-1α antibody

Fig 6.8

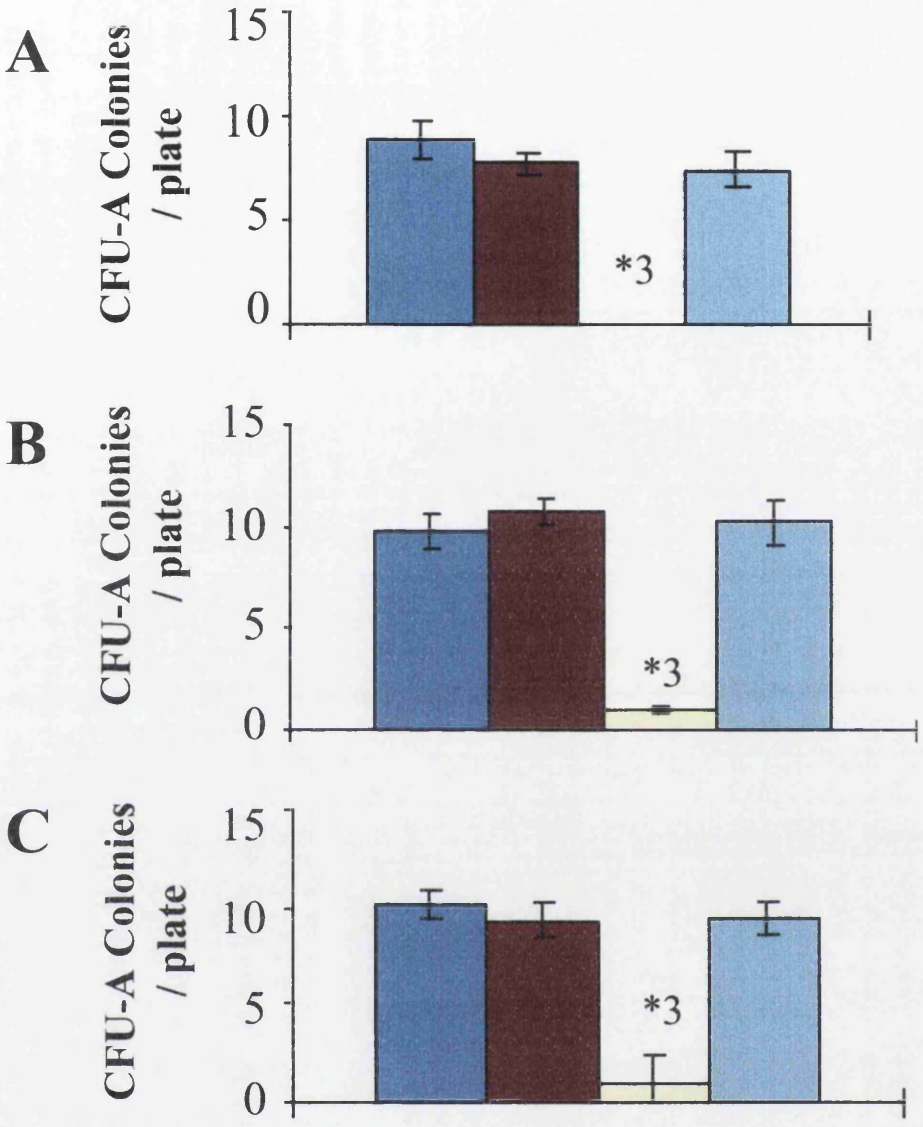


Figure 6.8 Colony Forming Unit Agar (CFU-A) analysis of the effect of anti-MIP-1α antibodies on the size of the CFU-A colonies

Unfractionated bone marrow was plated in a CFU-A assay containing 0.6, 6, 60 ng/ml rh M-CSF and these are represented in A, B and C respectively. The effects of MIP-1α at 100 ng/ml and of anti-MIP-1α antibody at 5 μg/ml on the size and number of the colonies were examined. The average colony production (+ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments Students T-test was performed comparing control (normal bone marrow) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Normal bone marrow
- Normal bone marrow 5 μg/ml of anti -MIP-1α antibody
- Normal bone marrow and 100 ng/ml of MIP-1α
- Normal bone marrow 100 ng/ml of MIP-1α plus 5 μg/ml of anti -MIP-1α antibody

to the assay. In all M-CSF concentrations 0.6, 6 and 60 ng/ml an average of approximately 10 colonies were produced, surprisingly no reduction in colony number was observed with high levels of M-CSF. These results are inconsistent with what has been previously observed, see figures 6.4 and 6.6. On the addition of the neutralising antibody there was a slight decrease in colonies in 0.6 and 60 ng/ml, neither effect is however statistically significant.

MIP-1α at 100 ng/ml reduced colony number to 1 or 0 ($p < 0.001$) and this was returned back to control levels for 0.6, 6 and 60 ng/ml of M-CSF (see figure 6.8). Furthermore, the inability of the neutralising antibody added to normal bone marrow to increase the CFU-A colony number over and above the level produced without the antibody in normal bone marrow suggests that high M-CSF may not induce the expression of MIP-1α.

6.3.5 The Effects of SCF and M-CSF on CFU-A Colony Formation from Normal or MIP-1α Null Mice Bone Marrow

In a further attempt to examine the expression of MIP-1α by high levels of SCF and M-CSF a CFU-A assay was set up comparing the CFU-A colony production from unfractionated bone marrow of a MIP-1α null mouse and a normal mouse, using various M-CSF and SCF concentrations. Figures 6.9 and 6.10, indicate that there is no significant difference in the number of CFU-A colonies produced by either bone marrow in any of the SCF or M-CSF concentrations and that MIP-1α can inhibit CFU-A colony production in both types of bone marrow equally well. However, it can be observed that again the higher levels of both rm SCF and rh M-CSF do reduce the average CFU-A colony number. If MIP-1α was expressed within the CFU-A colony in the assay containing 120 ng/ml SCF it might be expected that the bone marrow from the MIP-1α null mouse would produce more CFU-A colonies than the normal mouse. From figure 6.9C, a small increase in CFU-A numbers is observed but this is not significant again indicating that the high level of SCF or M-CSF do not produce the reduction in CFU-A colony formation by increasing the production of MIP-1α. It should be noted that as there is a great deal of redundancy within the chemokine family, and growth factors in general, it is possible that the inability of these cells to express MIP-1α may be overcome by another inhibitory molecule

Fig 6.9

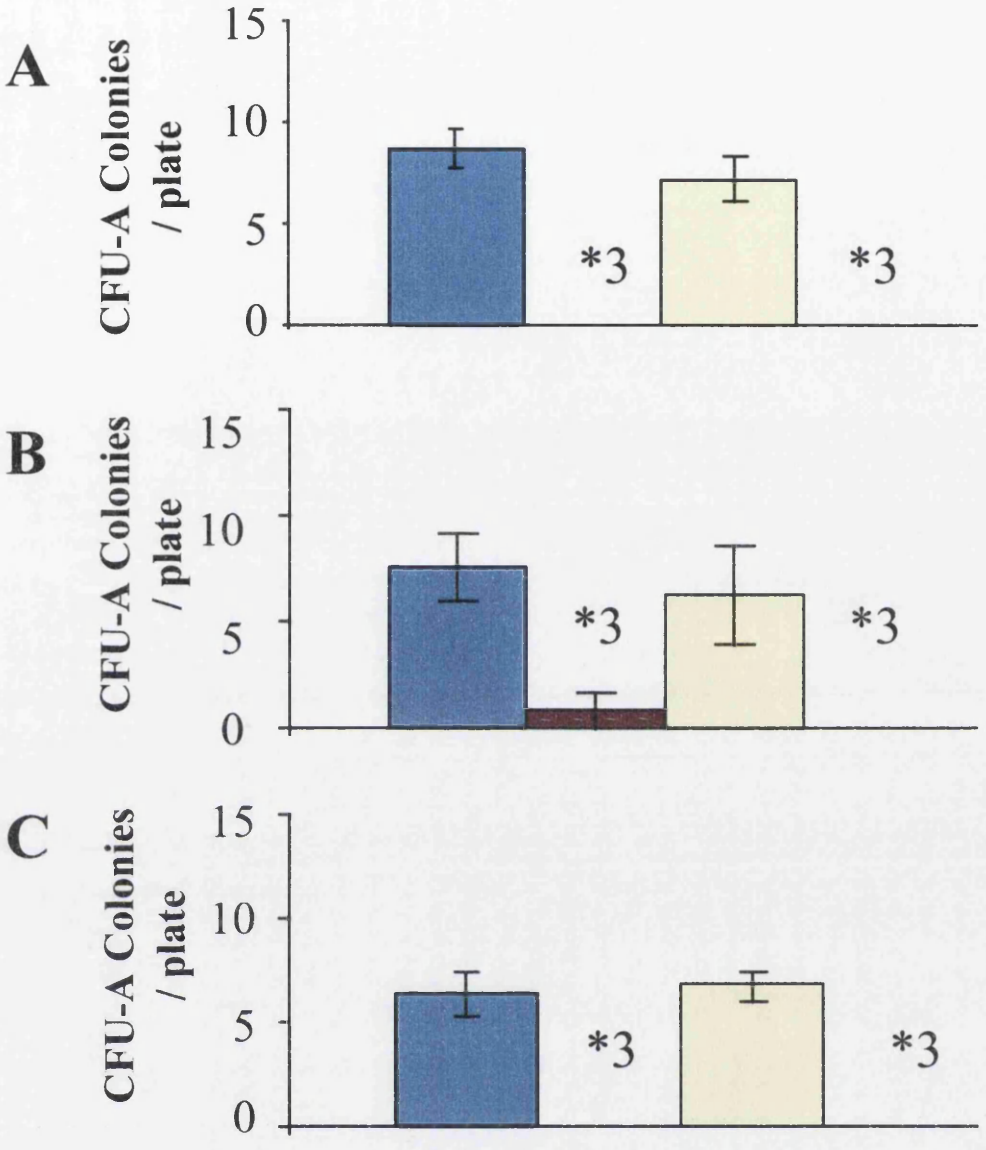


Figure 6.9 Comparison of the potential of bone marrow from a MIP-1α null mouse and a normal mouse to form CFU-A colonies in CFU-A assays containing 1.2, 12 and 120 ng/ml of rm SCF and in the absence and presence of 100 ng/ml of MIP-1α

Unfractionated bone marrow from a MIP-1α null mouse or a normal mouse was plated in a CFU-A assay containing 1.2, 12 and 120 ng/ml rm SCF in the presence or absence of 100 ng/ml of MIP-1α and can be observed in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (normal bone marrow) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Normal bone marrow
- Normal bone marrow and 100 ng/ml of MIP-1α
- Bone marrow from a MIP-1α Null mouse
- Bone marrow from a MIP-1α Null mouse and 100 ng/ml of MIP-1α

Fig 6.10

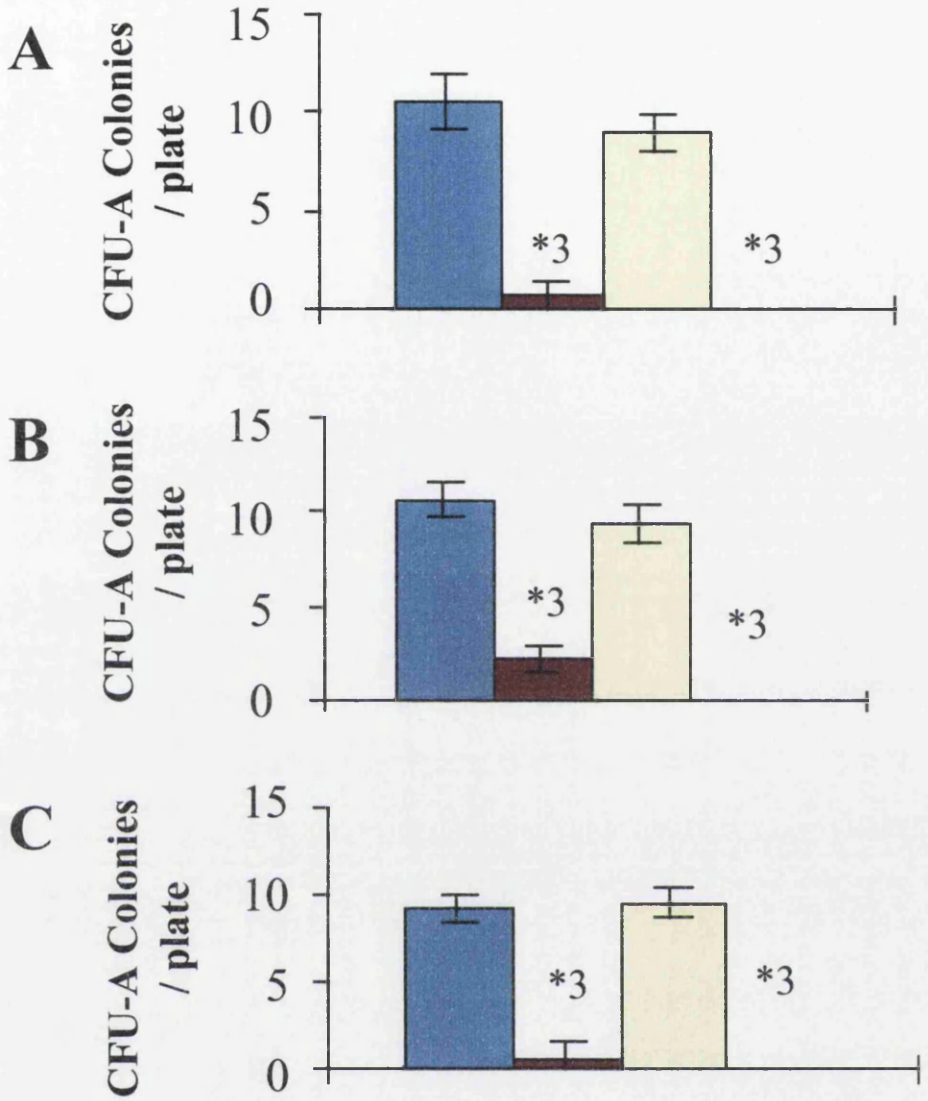


Figure 6.10 Comparison of the potential of bone marrow from a MIP-1α null mouse and a normal mouse to form CFU-A colonies in CFU-A assays containing 0.6, 6 and 60 ng/ml of rh M-CSF and in the absence and presence of 100 ng/ml of MIP-1α

Unfractionated bone marrow from a MIP-1α null mouse or a normal mouse was plated in a CFU-A assay containing 0.6, 6 and 60 ng/ml rh M-CSF in the presence or absence of 100 ng/ml of MIP-1α and these are represented in A, B and C respectively. The average colony production (+ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (normal bone marrow) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Normal bone marrow
- Normal bone marrow and 100 ng/ml of MIP-1α
- Bone marrow from a MIP-1α Null mouse
- Bone marrow from a MIP-1α Null mouse and 100 ng/ml of MIP-1α

taking its place eg MIP-1β or TGF-β. Further analysis of the expression of these other two inhibitory molecules is needed before firm conclusions can be reached.

6.3.6 The Effect of SCF on MIP-1α Expression in Monocytic Cell Lines

To further clarify the possible expression of MIP-1α by the CFU-A colonies, the possible induction of MIP-1α expression by SCF was examined in two monocytic cell lines, RAW and J774.2 cells. Monocytic cell lines were chosen due to the lack of macrophage cell lines and because monocytes are the precursor cells of macrophages.

Macrophages are the predominant cell type within the CFU-A colonies, see table 4, therefore it is possible that if SCF can induce the expression of MIP-1α within CFU-A colonies it will occur in these cells or their precursors. These cell lines were incubated for 48 hours in the presence or absence of 120 ng/ml of rm SCF and, samples of the conditioned media were removed at 0, 16, 24 and 48 hours and analysed for MIP-1α expression by ELISA. The baseline expression of MIP-1α in J774.2 and RAW cells is 5 and 42.8 ng/ml respectively. After 48 hours the expression of MIP-1α induced by rm SCF rose to 120 ng/ml in J774.2 cells and in 164 ng/ml RAW cells, (figure 6.11). However, this increase in the level of MIP-1α expression was not significantly different to that observed in cells treated with endotoxin free PBS, the same PBS used to reconstitute the rm SCF. Therefore, in this particular assay rm SCF does not induce the expression of MIP-1α in RAW or J774.2 cells over the levels induced by endotoxin free PBS.

6.3.7 The Effect of SCF on MIP-1α Expression in Bone Marrow Macrophages (BMM)

Using cell lines has its advantages however, it is not ideal therefore in an attempt to produce a situation that was nearer the conditions within the CFU-A colony, the analysis of MIP-1α production by bone marrow macrophages stimulated with rm SCF was examined. The expression of MIP-1α increased from 0 ng/ml at 0 hours to 300 ng/ml after 48 hours in untreated cells and this was also the case with cells treated with endotoxin free PBS (Figure 6.12). Bone marrow macrophages that were treated

Fig 6.11

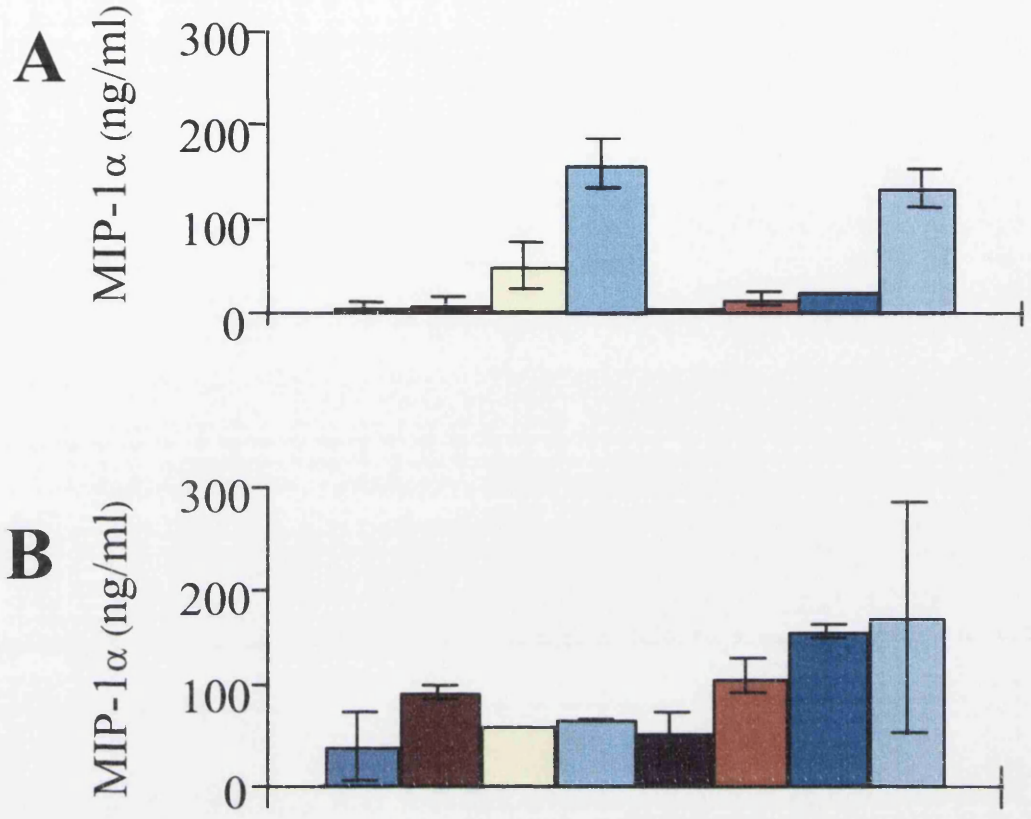


Figure 6.11 Analysis of the expression of MIP-1α by J774.2 and RAW cells upon stimulation with rm SCF

The expression of MIP-1α protein by ELISA was examined in J774.2 and RAW cells stimulated with rm SCF reconstituted in endotoxin free PBS and PBS for 48 hours and these are represented in A and B respectively. Samples were taken at 0, 16, 24 and 48 hours and analysed. Results represent a single experiment. The y-axis is the concentration of MIP-1α in ng/ml.

- 0 hours PBS
- 16 hours PBS
- 24 hours PBS
- 48 hours PBS
- 0 hours rm SCF
- 16 hours rm SCF
- 24 hours rm SCF
- 48 hours rm SCF

Fig 6.12

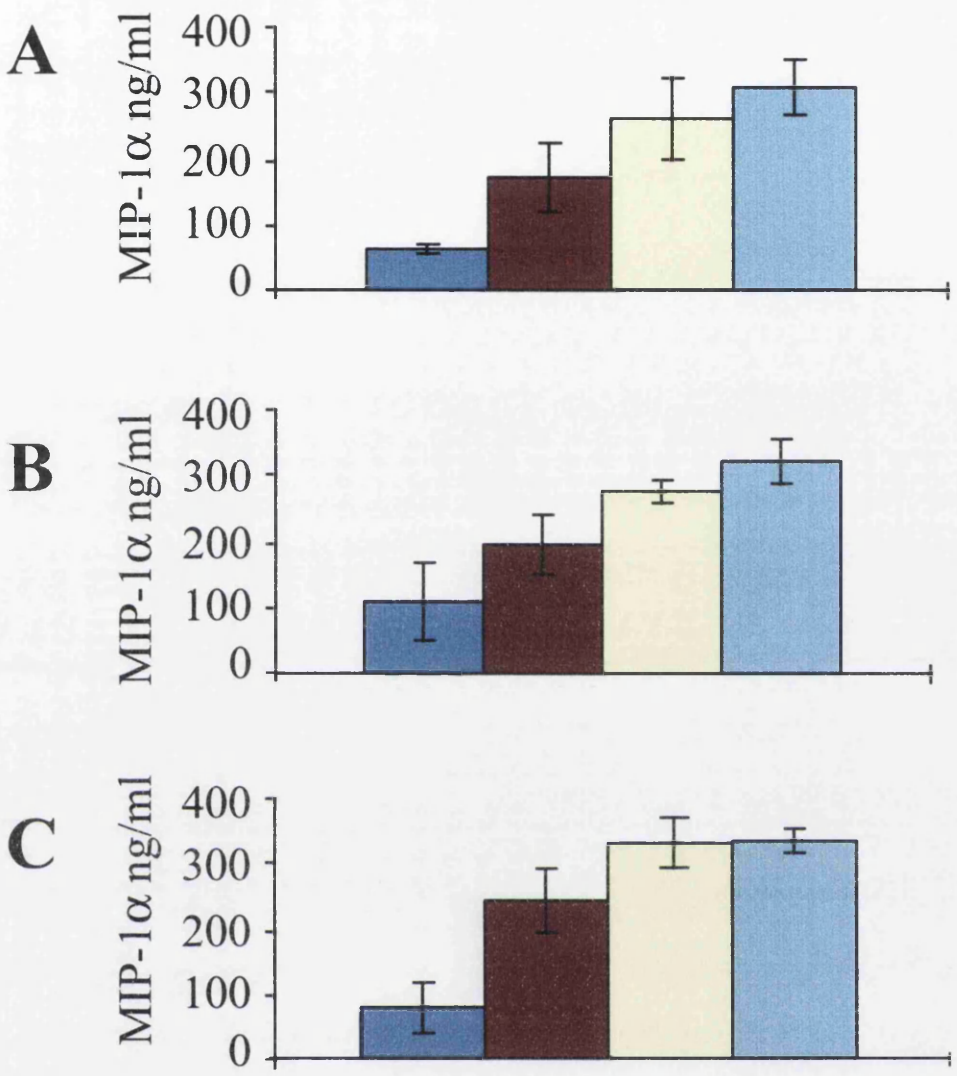


Figure 6.12 Analysis of the expression of MIP-1α by bone marrow macrophages cells upon stimulation with rm SCF

Bone marrow macrophage cells were made as described in the materials and methods. The bone marrow macrophages were either untreated or stimulated with rm SCF reconstituted in endotoxin free PBS and PBS for 48 hours and these are represented in A, B and C respectively. Samples were taken at 0, 16, 24 and 48 hours and analysed for the expression of MIP-1α protein by ELISA. Results represent a single experiment. The y-axis is the concentration of MIP-1α in ng/ml.

with rm SCF reconstituted in endotoxin free PBS produced the same level of MIP-1α as the other two treatments. As with the cell lines it seems that bone marrow macrophages do not express MIP-1α after stimulation with rm SCF.

In a final attempt to clarify whether 120 ng/ml SCF can induce expression of MIP-1α, total RNA was produced from un-stimulated bone marrow macrophages (BMM), BMM that were stimulated with endotoxin free PBS, or rm SCF reconstituted in endotoxin free PBS or GM-CSF. GM-CSF treated BMM were used as a positive control as Jarmin *et al* previously observed that GM-CSF induces MIP-1α expression in BMM (Jarmin *et al* 1999). 20 μg of total RNA was run on a gel and then immobilised onto a Hybond N membrane and was probed for the expression of MIP-1α using a radiolabelled MIP-1α specific probe (see methods section 4.2.3). The blot was then stripped and re-probed for the presence of β-actin as shown in figure 6.13. In lane 1, MIP-1α mRNA is expressed highly in the presence of GM-CSF as previously observed by Jarmin *et al.* This results also indicates that the treatment of bone marrow macrophages with rm SCF, lane 4, produces no increase in the expression of MIP-1α over the levels observed with bone marrow macrophages stimulated with PBS (lane 3) control or no treatment (lane 2). Therefore, the evidence indicates that whilst 120 ng/ml of SCF reduces CFU-A colony number it does not induce the expression of MIP-1α in CFU-A stem cells or the constituent cells of the CFU-A colony however this does not rule out the possibility of induction of other inhibitory growth factors other than MIP-1α. These further experiments indicate that SCF does not induce the expression of MIP-1α.

6.3.8 Summary

In the above sections the alteration of the concentration of M-CSF and SCF had no effect on the MIP-1α induced inhibition of CFU-A colony formation. However, upon increasing the concentration of both these growth factors it was observed that this had the effect of reducing the numbers of CFU-A colonies. This was hypothesised to be due to the increase in MIP-1α expression by the constituent cells of the CFU-A colony however, experiments using MIP-1α null mice and MIP-1α neutralising antibodies indicated that this was not the case. The inability of bone marrow

Fig 6.13

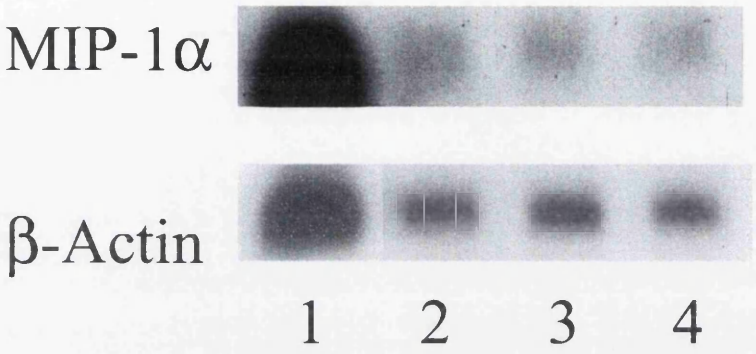


Figure 6.13 Northern blot analysis of Bone marrow macrophage expression of MIP-1α RNA upon stimulation with endotoxin free PBS or SCF

Bone marrow macrophages were made as described in the materials and methods. The bone marrow macrophages were either untreated or stimulated for 48 hours with endotoxin free PBS or rm SCF.

The upper panel is a northern blot of MIP-1α RNA expression by bone marrow macrophages. Lane 1 is a positive control that contains RNA from bone marrow macrophages stimulated with GM-CSF. Lane 2 contains RNA form un-stimulated bone marrow macrophages. Lane 3 contains RNA form bone marrow macrophages stimulated PBS and finally lane 4 contains RNA form bone marrow macrophages stimulated 100 ng/ml rm SCF. This blot was then stripped and reprobed for β-actin and this is represented by the lower panel.

macrophages to produce MIP-1α on incubation with SCF also provided further evidence to suggest that SCF did not reduce the CFU-A colony number by increasing the expression of MIP-1α. However, it is feasible that the activation of the tyrosine kinase signalling pathway by these growth factors may induce the expression of another inhibitory molecule such as MIP-1β or TGF-β. Therefore, further work is needed to further characterise this reduction in CFU-A colonies observed at high levels of both M-CSF and SCF.

CHAPTER 7

RESULTS 3 : The alteration of GM-CSF concentration on the inhibition of CFU-A colony formation by MIP-1α

7.1 Introduction

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) is a haemopoietic growth factor that stimulates the proliferation and differentiation of immature haemopoietic cells and regulates the functional activities of mature granulocytes, and macrophages, *in-vitro* and *in-vivo* (Kwon and Sckamato 1996). There are many cell types that have been reported to produce GM-CSF, these include haemopoietic cells such as T lymphocytes, macrophages, mast cells, B lymphocytes and natural killer cells (Nimer and Uchida 1995). Molecular cloning studies have indicated that the human and murine GM-CSF receptors are heterodimeric and are composed of an alpha (α) sub-unit, which binds to GM-CSF with low affinity, and a beta (β) sub-unit which is involved in signal transduction (Matsuguchi *et al* 1997). In humans the β sub-unit has no detectable binding affinity for GM-CSF, but forms a high affinity GM-CSF receptor with the α-sub-unit and both sub-units are members of the type-I cytokine receptor sub family (Gearing *et al* 1989, Hayashida *et al* 1990). Mice and humans have unique α-sub-units for the GM-CSF receptor however, they share the βc sub-unit with receptors for interleukin-3 (IL-3) and IL-5. In contrast to the human system, the mouse has two β sub-units, βc and β_{IL3} (Itoh *et al* 1990, Hayashida *et al* 1990) and like the human βc, the mouse βc is the common β sub-unit for mouse IL-3, GM-CSF and IL-5 receptors. Although β_{IL3}, has an extensive sequence homology with mouse βc, β_{IL3} does not form high affinity receptors with the α sub-units of murine IL-5 or GM-CSF. However, both βc and β_{IL3} interact equally well with mouse IL-3 α sub-unit in the presence of IL-3 to form high affinity IL-3 receptors (Hara *et al* 1992). In this part of the project the GM-CSF concentration was altered in an attempt to examine the ability of the β common signalling pathway to interact with the MIP-1α inhibitory pathway.

7.2 GM-CSF a Beta Common (βc) Signalling Growth Factor

7.2.1 The Effects of GM-CSF on CFU-A Colony Formation

GM-CSF is one of the main growth factors used to induce CFU-A colony formation. It was originally produced and added to the assay in the form of conditioned medium from AF1 19T cells. These cells are a NRK (rat fibroblast) cell line and produce GM-CSF after being transformed with the malignant hystiocytosis sarcoma virus (MHSV) (Franz *et al* 1989). As with M-CSF and SCF, the effects on CFU-A colony production were examined by altering the concentration of GM-CSF within the CFU-A assay. In the first experiment, rm SCF was added at 12 ng/ml and rh M-CSF at 6 ng/ml whereas, rm GM-CSF was either omitted or included at 0.0002, 0.002, 0.02, 0.2, 2 and 20 ng/ml. From the data in figure 7.1, it can be observed that with the omission of rm GM-CSF the CFU-A stem cells cannot produce CFU-A colonies. This is not surprising as GM-CSF is involved in the differentiation of monocyte/macrophage lineage and macrophages are the predominant cell type within the CFU-A colony. It was observed that with the omission of rm GM-CSF, a number of small colonies were produced (less than 0.5 mm in diameter) and these colonies did not show the characteristic morphology or size of CFU-A colonies. Further investigation is needed to examine the identity of these colonies, indeed, an ideal experiment would be picking these colonies out and staining them to identify the constuituent cells, however time restaints meant that these experiments were not performed. Alternatively it is possible that the lower concentration of GM-CSF may not allow the formation of CFU-A colonies by day eleven, and that by increasing the incubation time of the assay that CFU-A colony formation may occur at similar levels to controls. The addition of rm GM-CSF at 0.2 pg/ml did not allow CFU-A colonies to form, it was only after the addition of 2 pg/ml of rm GM-CSF that CFU-A colonies appeared giving an average of 3.4 colonies ($p < 0.001$). This number of CFU-A colonies was subsequently increased to 7.5, 9.4, 7.8 and 10 in the assays containing, 0.02, 0.2, 2 and 20 respectively.

7.2.2 The Effect of GM-CSF on the Constituent Cells of the CFU-A Colonies

The analysis of the effect of altering GM-CSF concentration on the cell types and

Fig 7.1

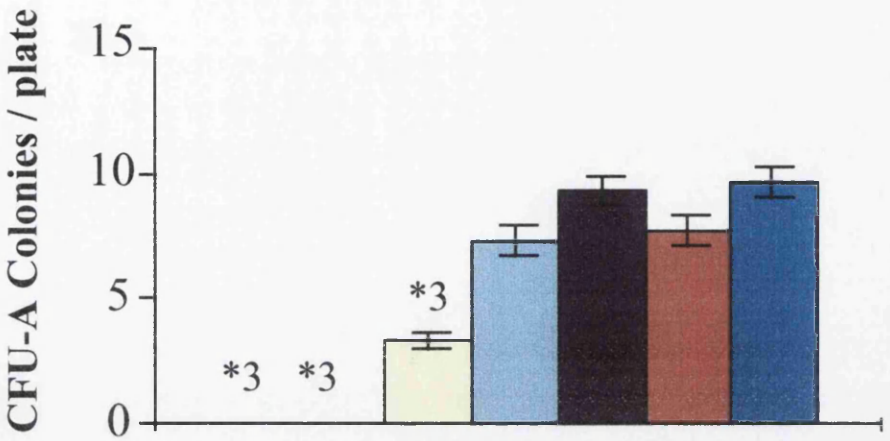


Figure 7.1 Colony Forming Unit Agar (CFU-A) analysis of the effect on the CFU-A colony production by increasing or reducing the rm GM-CSF concentration within the assay

A CFU-A assay was set up with normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and varying concentrations of rm GM-CSF. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing normal GM-CSF levels (0.2ng/ml) to all the other concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- 0 GM-CSF
- 0.0002 ng/ml GM-CSF
- 0.002 ng/ml GM-CSF
- 0.02 ng/ml GM-CSF
- 0.2 ng/ml GM-CSF
- 2 ng/ml GM-CSF
- 20 ng/ml GM-CSF

Table 6

Cell Numbers within CFU-A colonies

rm GM-CSF 0.02 ng/ml	19800
rm GM-CSF 0.02 ng/ml + MIP-1α	4400
rm GM-CSF 0.2 ng/ml	23200
rm GM-CSF 0.2 ng/ml + MIP-1α	6500
rm GM-CSF 2 ng/ml	24800
rm GM-CSF 2 ng/ml + MIP-1α	6900

Differential counts (500 cells)

	Macrophages	Granulocytes	Others
rm GM-CSF 0.02 ng/ml	494	4	2
rm GM-CSF 0.02 ng/ml + MIP-1α	498	0	2
rm GM-CSF 0.2 ng/ml	490	5	5
rm GM-CSF 0.2 ng/ml + MIP-1α	498	0	2
rm GM-CSF 2 ng/ml	476	22	2
rm GM-CSF 2 ng/ml + MIP-1α	487	11	2

Table 6 Analysis of cell number and morphology of the cells within the CFU-A colonies containing 0.02, 0.2 and 2 ng/ml of rm GM-CSF in the presence or absence of 100 ng/ml MIP-1α

CFU-A assays were set up containing a top layer of methylcellulose instead of the normal double agar assay. 10 colonies were picked from plates containing 100 ng/ml MIP-1α and 5 colonies from the plates that did not contain MIP-1α. These cells were washed and either cytospins were prepared and stained by Giemsa for morphology analysis or cell numbers were counted.

numbers within the CFU-A colonies was next examined. The results in table 6 indicate that the colonies are predominantly made up of macrophages. The only noticeable alteration in colony make up is an increase in granulocytes from 1 % in a normal CFU-A assay to 4.4 % in a CFU-A assay incubated with 2 ng/ml rm GM-CSF. Interestingly, the percentage of granulocytes in each CFU-A colony was subsequently reduced upon the further addition of 100 ng/ml of MIP-1α, from 4.4% to 2.2%. These results suggest that the increase in GM-CSF alters the constituent cells in the CFU-A colonies. The analysis of cell numbers within the CFU-A colonies indicate that there is a small reduction in cell numbers in colonies incubated in 0.2 ng/ml rm GM-CSF compared to that of 0.02 ng/ml of GM-CSF, 23200 to 19800. However, the opposite effect occurs upon adding 2 ng/ml of rm GM-CSF as this produces an increase in the constituent cells from 23200 to 24800. Furthermore, with the inclusion of 100 ng/ml of MIP-1α to each of the assays containing the various GM-CSF concentrations, it can be observed that there is an overall reduction in cell number by approximately 4 fold when compared to that of control CFU-A plates, see table 6.

7.2.3 The Effect of GM-CSF on MIP-1α Inhibition of CFU-A Colony Formation

In an attempt to investigate if altering the GM-CSF concentration could interfere with the inhibition of CFU-A colony formation by MIP-1α, the above GM-CSF titration was repeated with the inclusion of MIP-1α at concentrations ranging from 0.5 ng/ml to 100 ng/ml. At the normal levels of GM-CSF, 0.2 ng/ml figure 7.2C, it was observed that MIP-1α had the ability to inhibit CFU-A colony formation at 100 ng/ml. Although MIP-1α could also induce a statistically significant inhibition of CFU-A colony formation at 50 ng/ml, it was observed that MIP-1α was not as effective at inducing inhibition at this concentration compared to 100 ng/ml ($p < 0.001$). This was also true for CFU-A assays containing 2 and 20 ng/ml of GM-CSF. Within the assay containing 0.002 ng/ml of rm GM-CSF, it was observed that MIP-1α could produce inhibition of CFU-A colonies at concentrations as low as 5 ng/ml, $p < 0.05$, figure 7.2A. This indicates that it may be possible to enhance the ability of MIP-1α to inhibit the formation of CFU-A colony formation by reducing the GM-CSF concentration. Alternatively, this reduction in CFU-A colony numbers may be

Fig 7.2

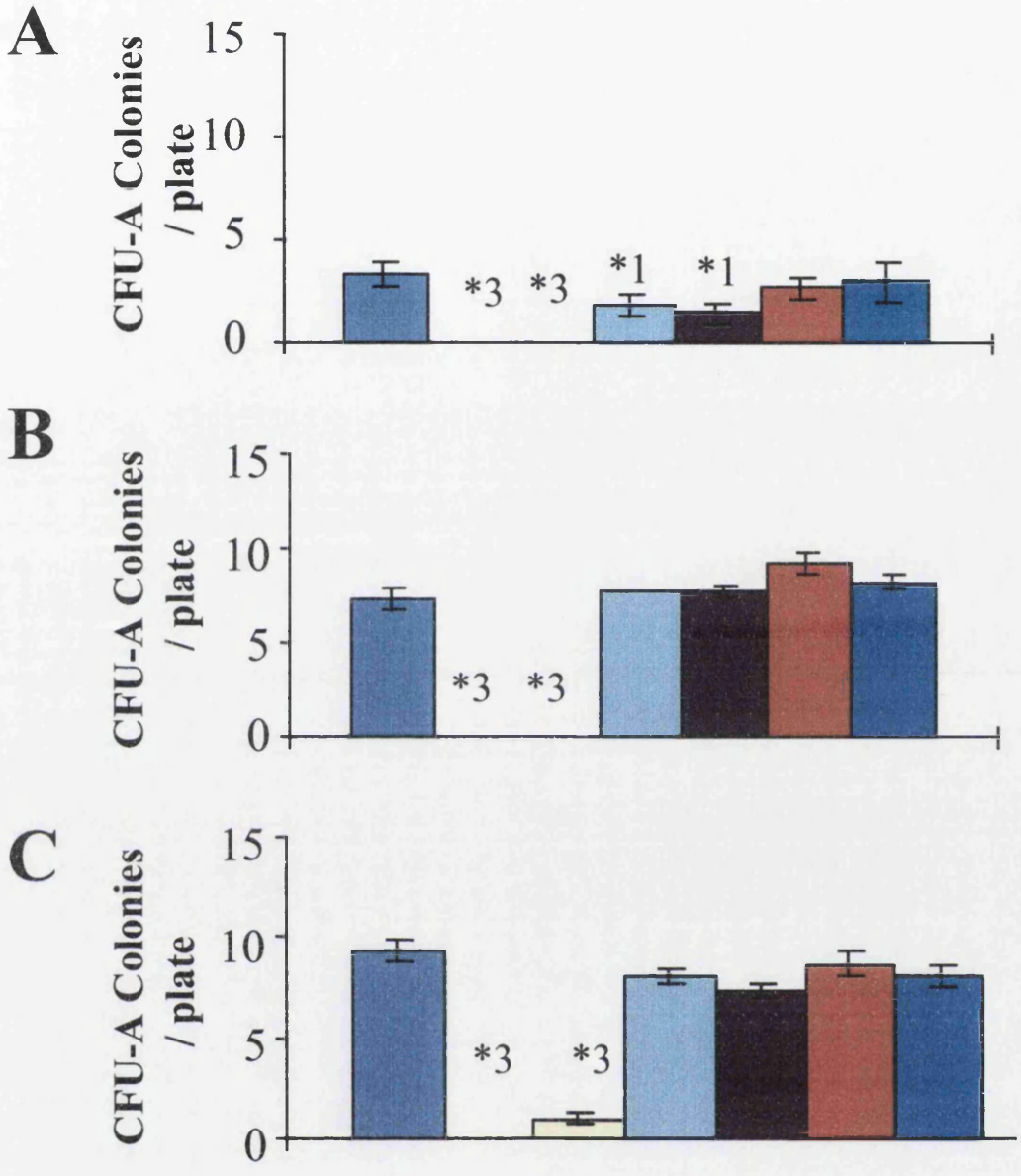


Figure 7.2 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 0.002, 0.02 and 0.2 ng/ml of rm GM-CSF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and either 0.002, 0.02 or 0.2 ng/ml of rm GM-CSF and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the other MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

Fig 7.2

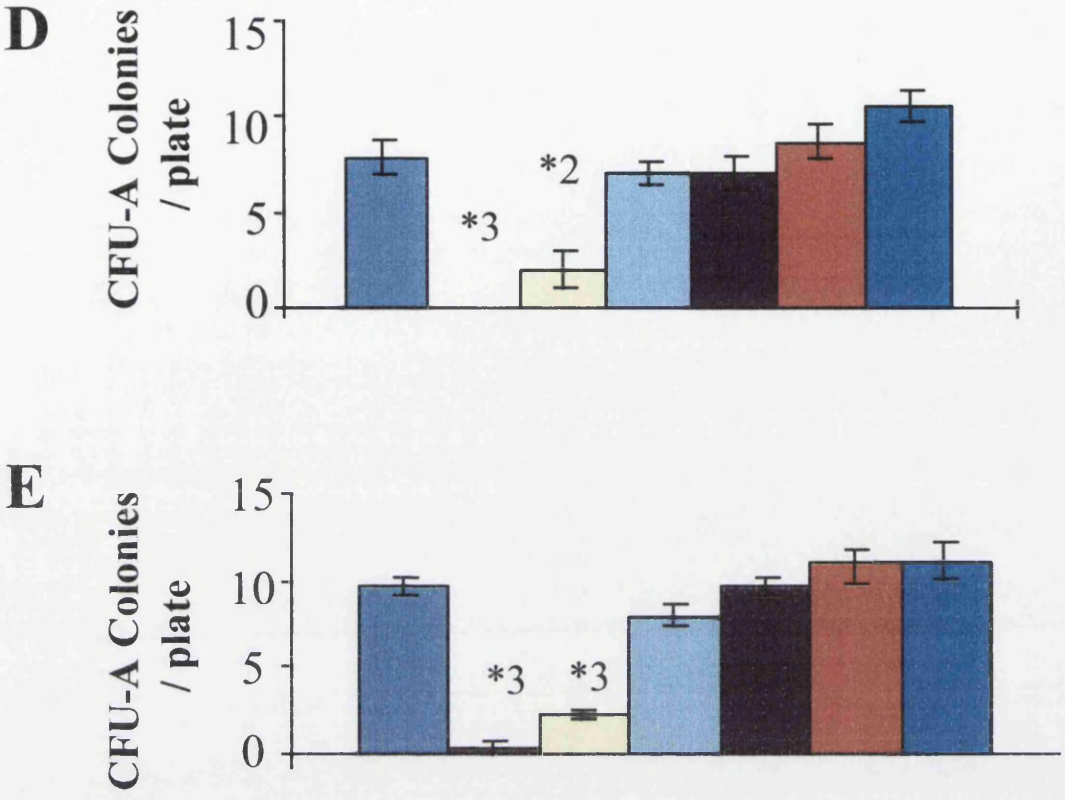


Figure 7.2 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production in the presence of 2 and 20 ng/ml of rm GM-CSF

MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and either 2 or 20 ng/ml of rm GM-CSF and these are represented in D and E respectively. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the other MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

due to this low level of GM-CSF not being able to support a similar level of survival, differentiation and proliferation needed for the formation of CFU-A colonies as observed in control assays. Indeed, on examining control CFU-A assays prior to day 11, small colonies that do not display the characteristic size of CFU-A colonies are observed and upon further incubation these colonies develop into CFU-A colonies. This has been proposed to be due to the inability of the growth factors to induce the normal levels of survival, differentiation and proliferation of the CFU-A stem cells in this smaller time scale. If this is the case it may explain the apparent ability of MIP-1α to inhibit CFU-A colony formation in CFU-A assays containing 0.002 ng/ml of GM-CSF. Further studies examining the effect of increasing the incubation time to day 14 or 20 may indicate that the low levels of GM-CSF are insufficient to allow formation of CFU-A colonies by day 11.

On further analysis of the CFU-A colonies produced in the above assay, an alternative colony morphology was observed in the assays containing 2 or 20 ng/ml of GM-CSF and 50 ng/ml of MIP-1α. These colonies were of similar size to normal CFU-A colonies, greater than or equal to 2 mm in diameter; however, they appeared to have a more defined edge than normal colonies. On staining CFU-A assays containing various GM-CSF concentrations with INT, and examining the plates from the side instead of from above, the colonies grown in 0.02, 0.2, 2 or 20 ng/ml GM-CSF, appear to be flat and are slightly embedded in the top layer of agar. Furthermore in assays containing 0.02, 0.2, 2 or 20 ng/ml GM-CSF + 100 ng/ml of MIP-1α, another colony morphology can be observed, see figure 7.3 and 7.4. On examining the colonies from 2 and 20 ng/ml GM-CSF in the presence of 50 ng/ml of MIP-1α compared to that of either no MIP-1α or indeed 100 ng/ml of MIP-1α, it was observed that these conditions resulted in another different colony shape. These colonies formed in the assay containing 2 or 20 ng/ml GM-CSF + 50 ng/ml of MIP-1α are similar in size to control CFU-A colonies. However, this is where the similarities stop, these alternative colonies are embedded in the two layers of the assay and from the side the colonies appear almost spherical, they also have a densely stained central region, a lighter stained exterior region and a densely stained outer edge.

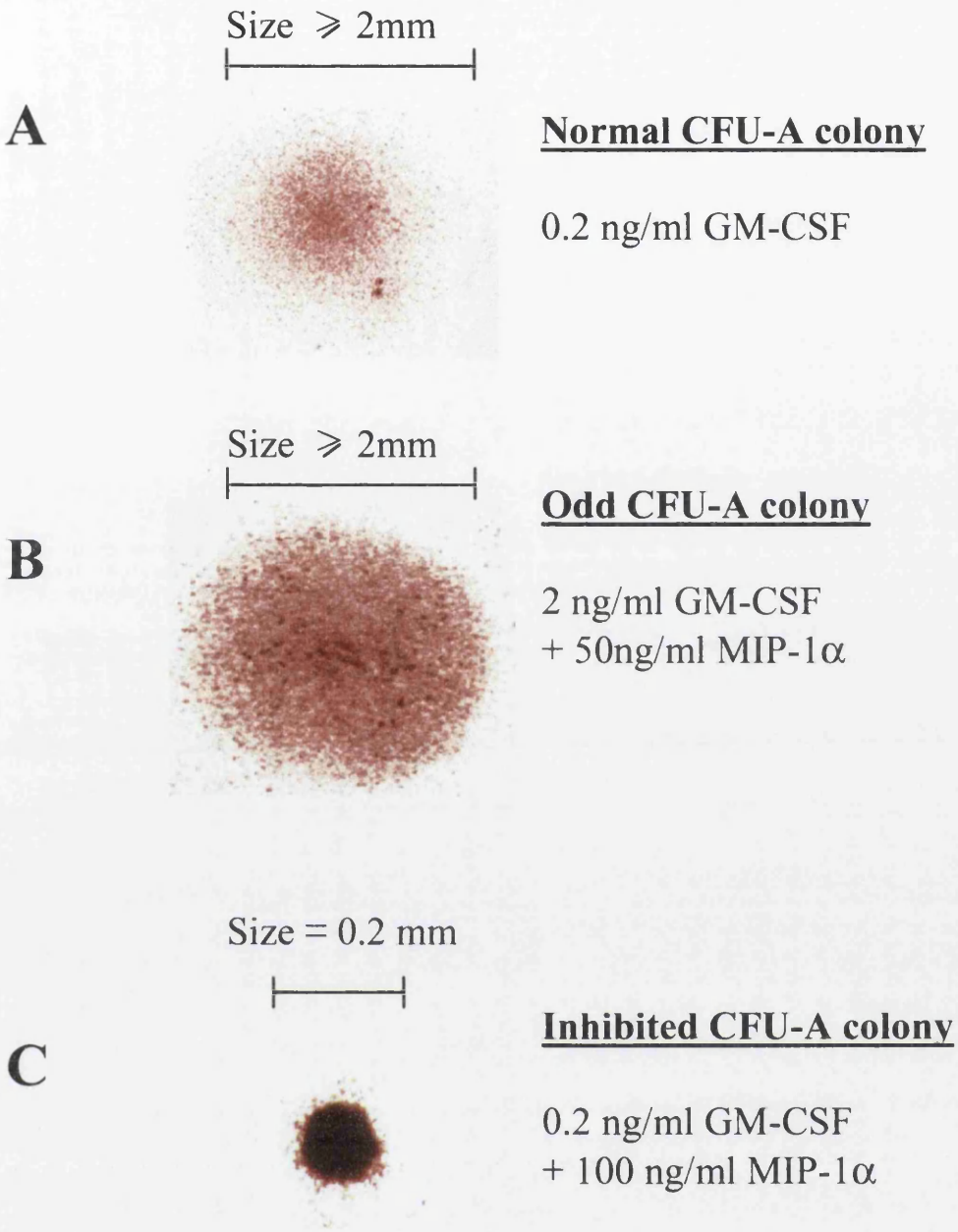


Figure 7.3 Photographic analysis of CFU-A colonies

Photographs of CFU-A colonies were produced as described in materials and methods. The CFU-A colonies were stained with INT and incubated overnight

A) Shows a photograph of a normal CFU-A colony produced in a CFU-A assay containing 0.2 ng/ml rm GM-CSF. Similar colonies are produced in assays containing 0.02, 2 and 20 ng/ml GM-CSF. The colony is greater than or equal to 2mm in size.

B) Shows a photograph of an odd shaped CFU-A colony produced in a CFU-A assay containing 2 ng/ml rm GM-CSF + MIP-1α 50 ng/ml. Similar colonies are produced in assays containing 20 ng/ml rm GM-CSF + MIP-1α 50 ng/ml or 2 ng/ml rm GM-CSF + rh HCC-1 1μg/ml or 20 ng/ml rm GM-CSF + rh HCC-1 1μg/ml. The colony is greater than or equal to 2mm in size.

C) Shows a photograph of an inhibited CFU-A colony produced in a CFU-A assay containing 0.2 ng/ml rm GM-CSF + MIP-1α at 100 ng/ml. Similar colonies are produced in assays containing 0.02, 2 and 20 ng/ml GM-CSF. The colony is less than 2 mm in size.

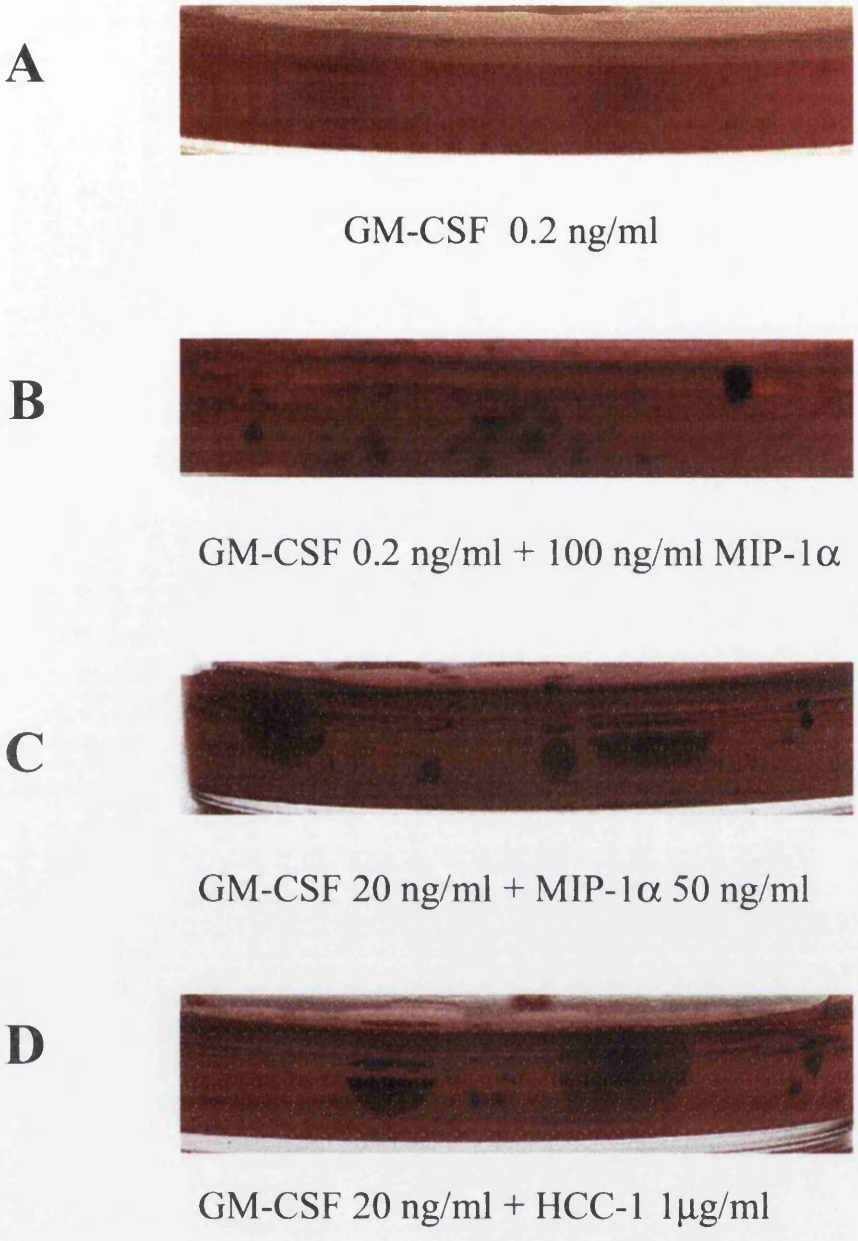


Figure 7.4 Photographic analysis of CFU-A colonies

Photographs of CFU-A colonies were produced as described in materials and methods. The CFU-A colonies were stained with May and Grunwald and Giemsa.

A) Shows a photograph of the side view of a plate that shows a representative colony shape which is produced in a CFU-A assay containing 0.2 ng/ml rm GM-CSF. Similar colonies are produced in assays containing 0.02, 2 and 20 ng/ml GM-CSF

B) Shows a photograph of the side view of a plate that shows a representative colony shape which is produced in a CFU-A assay containing 0.2 ng/ml rm GM-CSF + 100 ng/ml MIP-1α. Similar colonies are produced in assays containing 0.02, 2 and 20 ng/ml GM-CSF

C) Shows a photograph of the side view of a plate that shows a representative colony shape which is produced in a CFU-A assay containing 20 ng/ml rm GM-CSF + 50 ng/ml of MIP-1α. Similar colonies are produced in assays containing 2 ng/mlGM-CSF + 50 ng/ml of MIP-1α

D) Shows a photograph of the side view of a plate that shows a representative colony shape which is produced in a CFU-A assay containing 20 ng/ml rm GM-CSF + 1 μg/ml of HCC-1. Similar colonies are produced in assays containing 2 ng/mlGM-CSF + 1 μg/ml of HCC-1.

Jarmin *et al* previously observed that incubating bone marrow derived macrophages with GM-CSF induces the expression of various chemokines e.g. MIP-1α, MIP-1β, JE and MARC. Furthermore, they also observed that 10 ng/ml of GM-CSF could induce the expression of CCR-1 mRNA expression after 4 hours. Indeed, on analysis of surface expression of this receptor they observed a 130 % increase in CCR-1 expression from 20000 to 46000 receptors on bone marrow derived macrophages after incubation with GM-CSF. MIP-1α has been observed by a number of groups to be one of a number of chemokines that has the ability to interact with CCR-1 (Gao *et al* 1995, Post *et al* 1996). On this basis, it was hypothesised that the production of these alternatively shaped colonies in the current experiment may be due to the increased expression of CCR-1 by high levels of GM-CSF and the subsequent interaction with MIP-1α. From table 6, it can be observed that there is a 4 fold increase in granulocyte numbers in CFU-A assays containing 2 ng/ml GMCSF and this is reduced to a 2 fold increase upon the inclusion of 100 ng/ml MIP-1α compared to that of a normal CFU-A conditions. Therefore, it is feasible that CFU-A colonies grown in 2 or 20 ng/ml of rm GM-CSF + 50 ng/ml MIP-1α may have an increased number of granulocytes compared to that of CFU-A colonies grown in 0.2 ng/ml + 50 ng/ml MIP-1α. If so, this suggests that maybe the alteration in granulocyte numbers within these alternative colonies may be involved in their formation. It has been previously observed that CFU-GM colonies have a compact centre consisting largely of neutrophils and a dispersed halo of macrophages. Therefore, it is possible that the increase in granulocytes in these alternative CFU-A colonies occurs in the central region, if so this may explain the appearance of the tight central region. Furthermore, the increased level of GM-CSF may induce the expression of CCR1 on these granulocytes, and upon their interaction with MIP-1α, these granulocytes may become activated and produce a number of different cytokines or chemokines that may act to repel the macrophages from the centre of the colony. If this were to occur, this would explain the appearance of the leading edge observed in these colonies, see discussion.

7.2.4 Examination of the Alternative Colony Shape

Graham *et al* produced a variant of MIP-1α, which they designated HepMut due to its inability to bind to heparin. Functional analysis of HepMut indicated that it had retained the stem cell inhibitory property associated with the parent molecule however, it has an impaired ability to induce monocyte shape change or locomotion and did not show any detectable binding to the chemokine receptor CCR-1 (Graham *et al* 1996). In an attempt to examine the hypothesis that CCR-1 may be involved in the production of these alternative shaped colonies, an experiment comparing the effects of MIP-1α and HepMut on the CFU-A colony formation in assays with altered GM-CSF concentrations was set up, (see figure 7.5). MIP-1α at 100 and 50 ng/ml could significantly inhibit the formation of CFU-A colonies at all the GM-CSF concentrations tested, $p < 0.001$. As well as the observed inhibition by MIP-1α, HepMut at 100 ng/ml was able to inhibit the formation of CFU-A colonies significantly at 0.02 and 0.2 ($P < 0.001$). Although, HepMut did inhibit the CFU-A proliferation in the assays containing 2 ng/ml rm GM-CSF, this proved not to be statistically significant. HepMut could not significantly inhibit CFU-A formation at 50 ng/ml in any of the GM-CSF concentrations and like wise MIP-1α and HepMut did not produce any inhibition at concentrations lower than 50 ng/ml in any of the assays. On analysis of the morphology of the colonies, it was observed that the HepMut colonies were similar to the colonies observed in the control plates, and that they did not resemble the alternative shaped colonies produced in 2 or 20 ng/ml GM-CSF + 50 ng/ml MIP-1α assays. Both MIP-1α and HepMut have been observed to inhibit the formation of CFU-A colonies however, only MIP-1α can induce the production of the alternative colonies suggesting that the inhibitory property of MIP-1α does not play a part in the creation of these alternative colonies. As HepMut can not bind to CCR-1 and can not produce these alternative colonies this may suggest that the formation of these colonies is due to the interaction between MIP-1α and CCR-1. Furthermore, these results indicate that the interaction between CCR1 and MIP-1α may be involved in the activation of an alternative signalling pathway other than the pathway used to produce the inhibition of the CFU-A colonies.

Fig 7.5

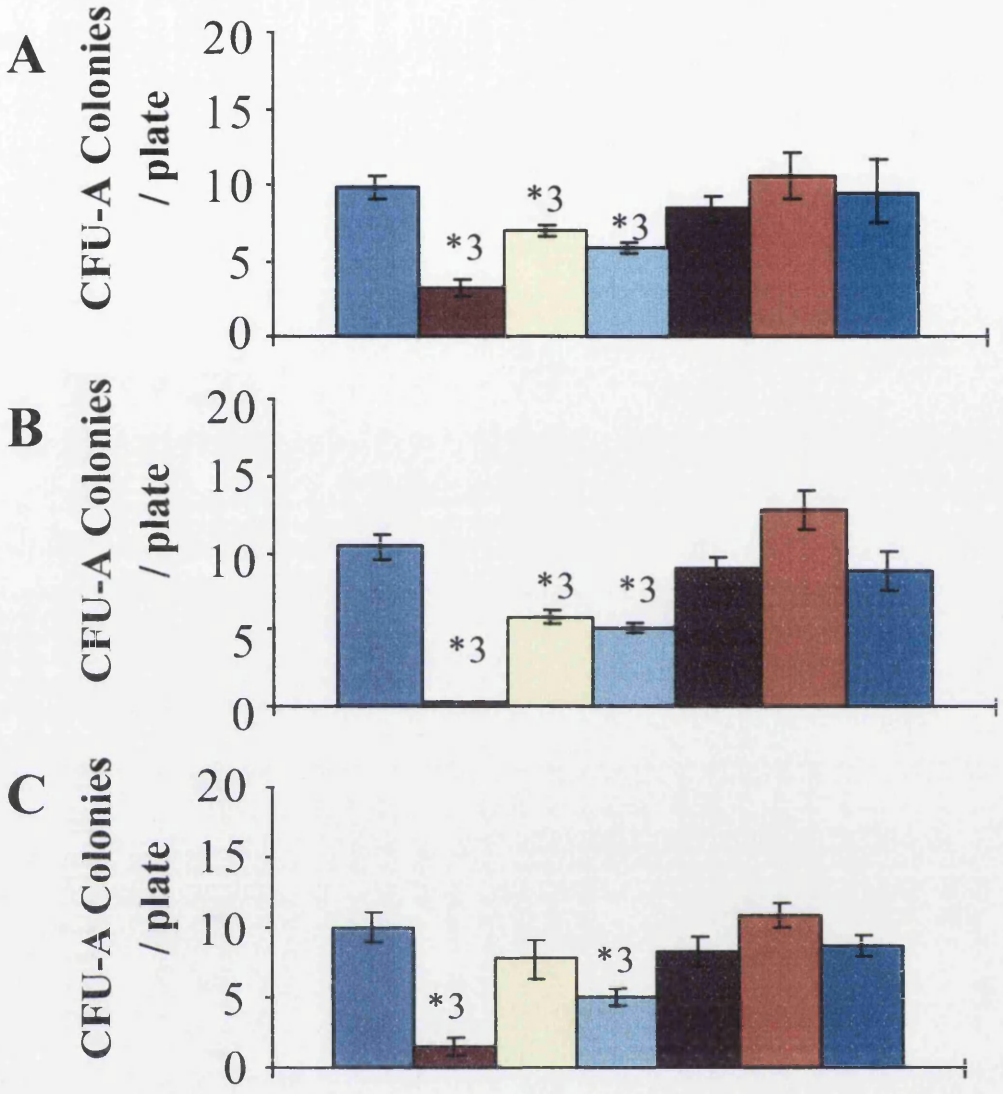


Figure 7.5 Colony Forming Unit Agar (CFU-A) analysis of the effect of HepMut a non heparin binding variant of MIP-1α on the CFU-A colony production in the presence of 0.02, 0.2 and 2 ng/ml of rm GM-CSF

HepMut and MIP-1α at 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and either 0.02, 0.2 or 20 ng/ml of rm GM-CSF and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 100 ng/ml HepMut
- 50 ng/ml MIP-1α
- 50 ng/ml HepMut
- 10 ng/ml MIP-1α
- 10 ng/ml HepMut

Fig 7.6

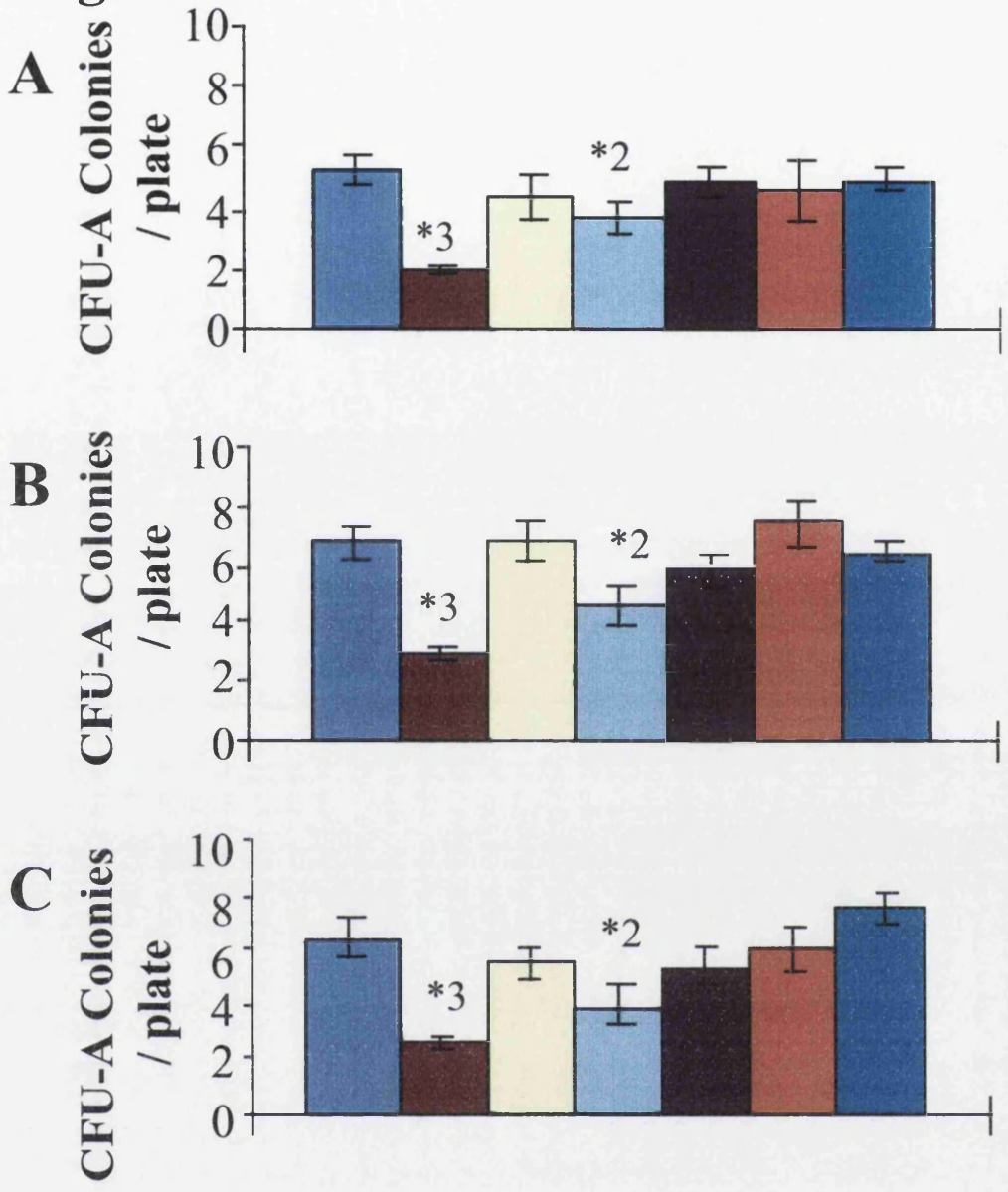


Figure 7.6 Colony Forming Unit Agar (CFU-A) analysis of the effect of mMCP-3 a CCR-1 ligand on the CFU-A colony production in the presence of 0.02, 0.2 and 2 ng/ml of rm GM-CSF

MCP-3 and MIP-1α at 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and either 0.02, 0.2 or 2 ng/ml of rm GM-CSF and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

7.2.5 The Effects of High Levels of GM-CSF and other CCR-1 Ligands on CFU-A Colony Formation

In a further attempt to examine the role of CCR1 in the formation of these alternative colonies at high GM-CSF concentration and 50 ng/ml of MIP-1α, the effects of two other CCR-1 ligands were examined, neither of which have been previously shown to inhibit CFU-A colony formation. The data in figure 7.6 indicates, that MIP-1α at 100 and 50 ng/ml can inhibit the CFU-A colony formation at all GM-CSF concentrations, ($p < 0.001$ and $p < 0.05$), see figure 7.6. However, rm MCP-3 can not inhibit CFU-A colony formation at 100, 50 or 10 ng/ml and did not induce the formation of the alternatively shaped colonies (data not shown).

Tsou *et al* using a panel of cloned chemokine receptors revealed that rh HCC-1 could bind to and induce signalling through hCCR-1 (Tsou *et al* 1998). With a few exceptions most human chemokines that signal through a particular human chemokine receptor have been observed to signal through its murine counterpart. To date no murine homologue of the human HCC-1 has been identified therefore, the human protein was used to examine the potential for hHCC-1 to produce these alternative shaped colonies. This was examined by the addition of rh HCC-1 to a CFU-A assay containing various concentrations of GM-CSF, (figure7.7). In this assay it was observed that again MIP-1α could inhibit CFU-A colonies at 100 and 50 ng/ml and although 100 ng/ml of rh HCC-1 could reduce the number of CFU-A colonies produced at all GM-CSF concentrations this was not statistically significant, (figure 7.7). Furthermore, on examination of the morphology of the colonies under the conditions where the alternatively shaped colony morphology was produced it was observed that hHCC-1 could not induce the formation of these alternative shaped colonies. Human HCC-1 has been observed to compete with rm MIP-1α for binding to mCCR-1 transfected cell lines with a markedly reduced affinity (IC_{50} = 93 nM compared to that of 1.3 nM for MIP-1α), and hHCC-1 is also 100 fold less efficient at inducing chemotaxis compared to mMIP-1α in freshly isolated human monocytes (Tsou *et al* 1998). Therefore, it is possible that higher concentrations of rh HCC-1 may in fact produce the formation of these alternatively shaped colonies. In figure 7.8, a CFU-A assay was set up containing 20 ng/ml GM-CSF with the addition of 100, 50 ng/ml of MIP-1α, 1μg/ml of rm RANTES, rm MCP-3 or rh HCC-1. MIP-1α as expected inhibited the colony formation at 100 and 50 ng/ml and the alternative

Fig 7.7

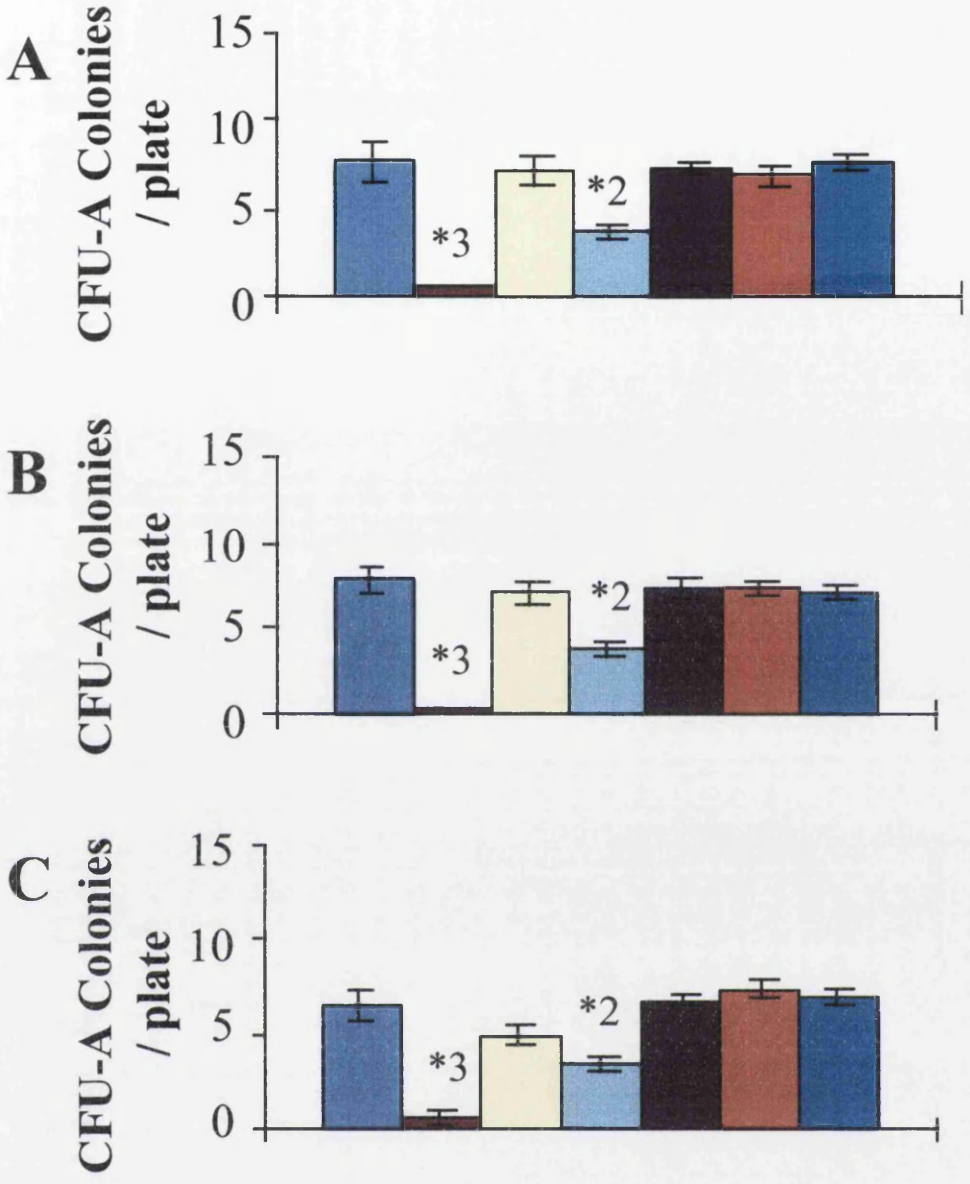


Figure 7.7 Colony Forming Unit Agar (CFU-A) analysis of the effect of hHCC-1 a CCR-1 ligand on the CFU-A colony production in the presence of 0.02, 0.2 and 2 ng/ml of rm GM-CSF

HCC-1 and MIP-1α at 10, 50 and 100 ng/ml was added to the CFU-A assay containing normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and either 0.02, 0.2 or 2 ng/ml of rm GM-CSF and these are represented in A, B and C respectively. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 100 ng/ml HCC-1
- 50 ng/ml MIP-1α
- 50 ng/ml HCC-1
- 10 ng/ml MIP-1α
- 10 ng/ml HCC-1

Fig 7.8

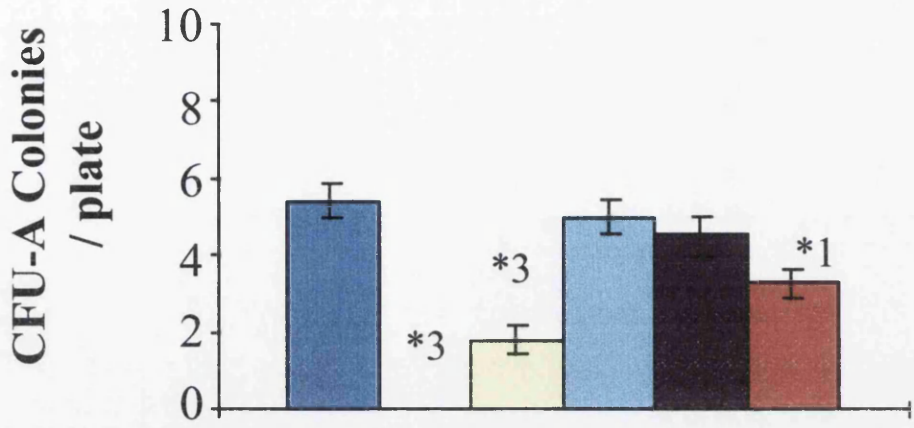


Figure 7.8 Colony Forming Unit Agar (CFU-A) analysis of the effect of mMIP-1α, mMCP-3, hHCC-1 and RANTES on the CFU-A colony production in the presence 20 ng/ml of rm GM-CSF

MIP-1α was added to the assay to give a final concentration of 50 or 100 ng/ml whereas the other CCR-1 ligands had a final concentration of 1μg/ml. The CFU-A assay contains normal levels of rh M-CSF (6 ng/ml), rm SCF (12 ng/ml) and 20 ng/ml of rm GM-CSF. (100 fold higher than normal levels of 0.2 ng/ml). The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from two replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 1μg/ml RANTES
- 1μg/ml MCP-3
- 1μg/ml HCC-1

colony shape was observed at 50 ng/ml ($p < 0.001$). Murine RANTES and mMCP-3, at 1 μg/ml, did not inhibit CFU-A colony formation nor did they produce the alternatively shaped colonies. On searching the literature there is a limited amount of binding affinity data relevant to the interaction of these murine ligands with mCCR-1 however, there is data to suggest that rm RANTES is a low affinity ligand for mCCR-1, if this is also true for mMCP-3 and rh HCC-1 this may explain why low concentrations of these CCR-1 ligands do not induce the formation of the alternative colonies. If this is the case, the addition of a substantially higher concentration of both chemokines will enable these low affinity ligands to interact with mCCR-1, ruling out the low affinity of these ligands as an explanation of why they do not induce the formation of these alternative shaped colonies. Unlike rm RANTES and rm MCP-3, the addition of 1 μg/ml of rh HCC-1 to the CFU-A assay produced a small but statistically significant inhibition of CFU-A colony formation ($p < 0.05$). This suggests that rh HCC-1 can inhibit CFU-A colony formation and that this high concentration may be needed to allow a weak interaction with an unknown inhibitory receptor. However, it is also possible that this reduction in CFU-A colonies is due to the very high concentration of rhHCC-1 being toxic to either the CFU-A stem cells or the mature cells of the CFU-A colony. Alternatively, the formation of these alternative colonies could be due to a non-specific interaction with this high level of hHCC-1. On the analysis of the colony shape it was observed that the colonies produced in high levels of rh HCC-1 were similar in shape to that observed with 2 or 20 ng/ml GM-CSF + 50 ng/ml of MIP-1α, see figure 7.3 and 7.4.

Therefore, from the above observations it seems that the appearance of these alternative shaped colonies may be due initially to the increased expression of CCR-1 by high levels of GM-CSF and the subsequent interaction between this receptor and some of its ligands. Interestingly, MIP-1α and rh HCC-1 could not only induce the formation of these alternative shaped colonies they were also observed to inhibit the formation of CFU-A colonies. Therefore, it may be that the interaction between two different signalling pathways induced through distinct receptors may play a role in the formation of these alternatively shaped colonies.

7.2.6 CCR1 and its possible role in the Alternative Colony Shape

In chapter 9, total RNA produced from lineage depleted bone marrow cells, previously expanded by treatment with rm SCF and rh IL-11 for 6 days, were examined for the expression of CC chemokine receptors. It was observed that CCR-1 was up-regulated at the RNA level in lineage depleted expanded bone marrow cells compared to non expanded lineage depleted bone marrow cells, (figure 9.6). If this up regulation of CCR-1 transcripts is the same at the protein level, and if as hypothesised CCR-1 is involved in the production of these alternative colonies, it would be expected that plating these expanded CFU-A stem cells in a normal CFU-A assay in the presence of MIP-1α would produce these alternative shaped colonies. To examine if this was the case, expanded stem cells were plated in a CFU- A assay containing a titration of MIP-1α and compared to non expanded stem cells, in the presence or absence of high levels of GM-CSF. In this experiment the expanded CFU-A colonies appeared to have a defined edge and looked similar to the alternative shaped colonies referred to before, at all concentrations of MIP-1α used except 100ng/ml (data not shown). Therefore, it seems that the expansion of CFU-A stem cells can produce conditions that allow the formation of colonies that are similar to the alternatively shaped colonies produced in 2 or 20 ng/ml GM-CSF + 50 ng/ml of MIP-1α. All the evidence thus far seems to point to a link between the formation of these alternative shaped colonies and the interaction of MIP-1α with the chemokine receptor CCR-1. The analysis of the ability of bone marrow from CCR-1 null mice to produce these alternative colonies would provide conclusive evidence that CCR-1 is the predominant receptor involved in this process. However, within the time scale of this project this type of experiment could not be performed.

7.2.7 Summary

In summary, the decrease in GM-CSF concentration reduces the formation of the CFU-A colonies confirming that GM-CSF is an essential growth factor for the production of CFU-A colonies. The addition of MIP-1α to assays containing low levels of GM-CSF was observed to reduce the number of CFU-A colonies produced. This may be inhibition but more probably is due to the lack of differentiation of the CFU-A stem cells as discussed above. The increase in GM-CSF concentration within the CFU-A assays had no effect on the inhibition of the formation of CFU-A colonies

by MIP-1α however, it was observed that there was an increase in the number of neutrophils within the CFU-A colonies. Although increased levels of GM-CSF did not interfere with the inhibition of the CFU-A colony formation by MIP-1α it did however, alter the colony shape and this was observed in assays containing 2 and 20 ng/ml of GM-CSF + 50 ng/ml of MIP-1α. The data presented above suggests that the formation of these alternative colonies involves the interaction between the increased levels of CCR1 and MIP-1α, see discussion.

CHAPTER 8

RESULTS 4 : The effect of the addition of gp130 signalling proteins on the ability of MIP-1α to inhibit CFU-A colony formation

8.1 Introduction

Interleukin-11 (IL-11) was first cloned from a primate bone marrow derived stromal cell line PU34, as a factor that stimulated proliferation of an IL-6 dependant plasmacytoma cell line (Paul *et al* 1990). The human and murine IL-11 receptors have been identified and share 82 % sequence homology and they have two independent sub-units, a ligand binding sub-unit and a signal-transducing sub-unit. The signalling sub-unit, gp130, is not unique to the IL-11 receptor and is a characteristic feature of a family of cytokines that have been identified by their ability to use this particular sub-unit to induce a signal within the cell. Oncostatin M, ciliary neurotrophic factors (CNTF), interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF), are further examples of members of this family (Taga and Kishimoto 1997). IL-6 can support the growth and differentiation of both granulocyte-macrophage and megakaryocyte progenitors (Kimura *et al* 1990, Hirano *et al* 1996). Furthermore, IL-6 has been observed to induce murine multi-lineage cells to exit G_0 and enter cell cycle, where they can be acted upon by various growth factors e.g. IL-3 (Mushashi *et al* 1991). Leukaemia inhibitory factor can also act in combination with IL-3 and these growth factors were observed to enhance the survival and proliferative response of primitive blast cells. Thus, IL-11, IL-6 and LIF have been implicated as stimulatory proteins for early hematopoietic stem/progenitor cells, and all signal through the gp130 signalling pathway. Therefore, IL-6, IL-11 and LIF were individually added to the CFU-A assay in an attempt to examine the ability of the gp130-signalling pathway to interact with the MIP-1α inhibitory pathway.

8.2 Glycoprotein 130 (gp-130) Signalling Cytokines

8.2.1 The Effect of IL-11 and IL-6 on CFU-A Colony Formation

In the first experiment, the effect of IL-11 and IL-6 on the formation of CFU-A colonies was examined. Various concentrations of rh IL-11 and rh IL-6, ranging from

500 pg/ml to 100 ng/ml, were added to a CFU-A assay containing 12 ng/ml of rm SCF, 6 ng/ml of rh M-CSF and 0.2 ng/ml of rm GM-CSF. The addition of 0.5 ng/ml of rh IL-11 produced an increase in CFU-A colony numbers however this was not statistically significant. Upon the further increment of rh IL-11 concentration to 1 or 5 ng/ml, no further increase or decrease in colony numbers was observed, (figure 8.1). However, the addition of 100, 50 and 10 ng/ml of rhIL-11 reduced the CFU-A colony numbers from 9.47 to 4.8, 6.0 and 4.9 respectively ($p<0.001$). Unlike rh IL-11, the addition of 0.5 ng/ml of rh IL-6 to a CFU-A assay did not produce an increase in CFU-A colonies, and on the subsequent increase of rh IL-6 concentration to 1 ng/ml no alteration in colony numbers was observed. However, on the addition of 5, 10, 50 and 100 ng/ml of rh IL-6, a decrease in CFU-A colony numbers was observed from 6.25 to 3.8, 1.3, 0.8 and 0.6 respectively, $p < 0.05$ and $p < 0.001$ (figure 8.2). These results indicate that both rh IL-11 and rh IL-6 have the effect of reducing the formation of CFU-A colonies and that rh IL-6 is more effective than rh IL-11.

8.2.2 The Effect of IL-11 and IL-6 on MIP-1α Inhibition of CFU-A Colonies

To examine the effect of rh IL-11 and rh IL-6 on the inhibition of the CFU-A stem cells by MIP-1α, a subsequent assay containing a titration of rh IL-11 or rh IL-6 and MIP-1α at 1, 10 and 100 ng/ml was set up. In figure 8.3, it can be observed that 100 ng/ml of MIP-1α can not only significantly inhibit the formation of CFU-A colonies in the absence of rh IL-11, it can also significantly inhibit the formation of CFU-A colonies in the presence of the various concentrations of rh IL-11. No further inhibition of CFU-A colonies were observed at concentrations lower than 100 ng/ml of rm MIP-1α. With the inclusion of 100 ng/ml rm MIP-1α in the CFU-A assays containing 0.5, 1, 5, 10, 50 and 100 ng/ml of rh IL-6, it can be observed that the formation of CFU-A colonies is totally inhibited ($p < 0.001$) in all concentrations of rh IL-6 (figure 8.4). Interestingly, in CFU-A assays containing 50, 10 and 5 ng/ml of rh IL-6, the inhibition of CFU-A colonies also occurred at 10 ng/ml of rm MIP-1α. This is a concentration of rm MIP-1α that does not normally induce inhibition of CFU-A colony formation, therefore, it seems that rh IL-6 and rm MIP-1α are having an additive effect in reducing the formation of CFU-A colonies.

Fig 8.1

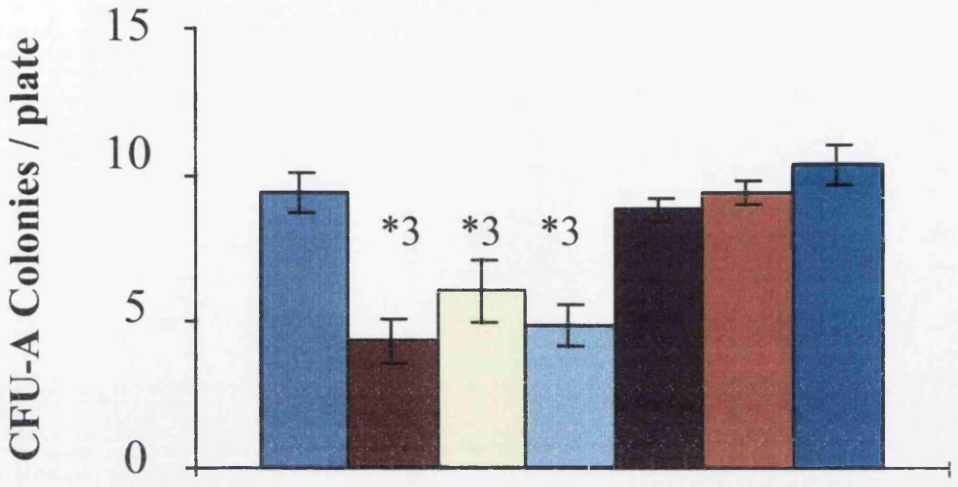


Figure 8. 1 Colony Forming Unit Agar (CFU-A) analysis of the effect of Interleukin-11 on the CFU-A colony production

Interleukin-11 at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to a normal CFU-A assay containing 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml rh IL-11
- 50 ng/ml rh IL-11
- 10 ng/ml rh IL-11
- 5 ng/ml rh IL-11
- 1 ng/ml rh IL-11
- 0.5 ng/ml rh IL-11

Fig 8.2

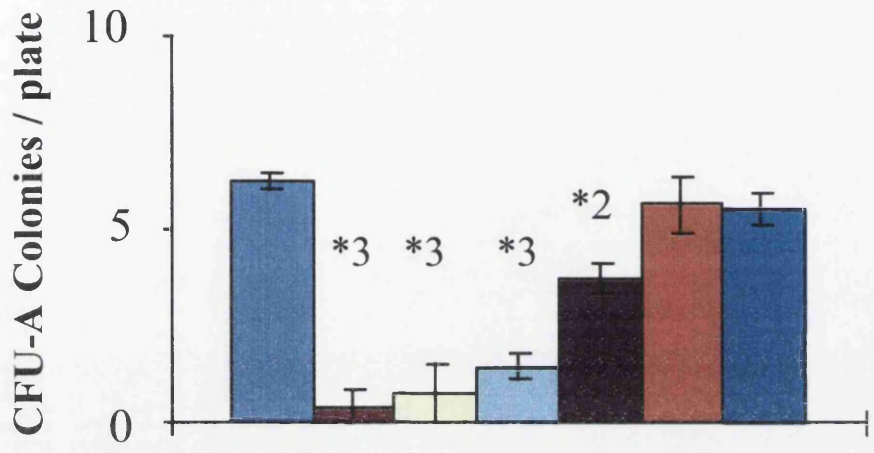


Figure 8. 2 Colony Forming Unit Agar (CFU-A) analysis of the effect of Interleukin-6 on the CFU-A colony production

Interleukin-6 at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to a normal CFU-A assay containing 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml IL-6
- 50 ng/ml IL-6
- 10 ng/ml IL-6
- 5 ng/ml IL-6
- 1 ng/ml IL-6
- 0.5 ng/ml IL-6

Fig 8.3

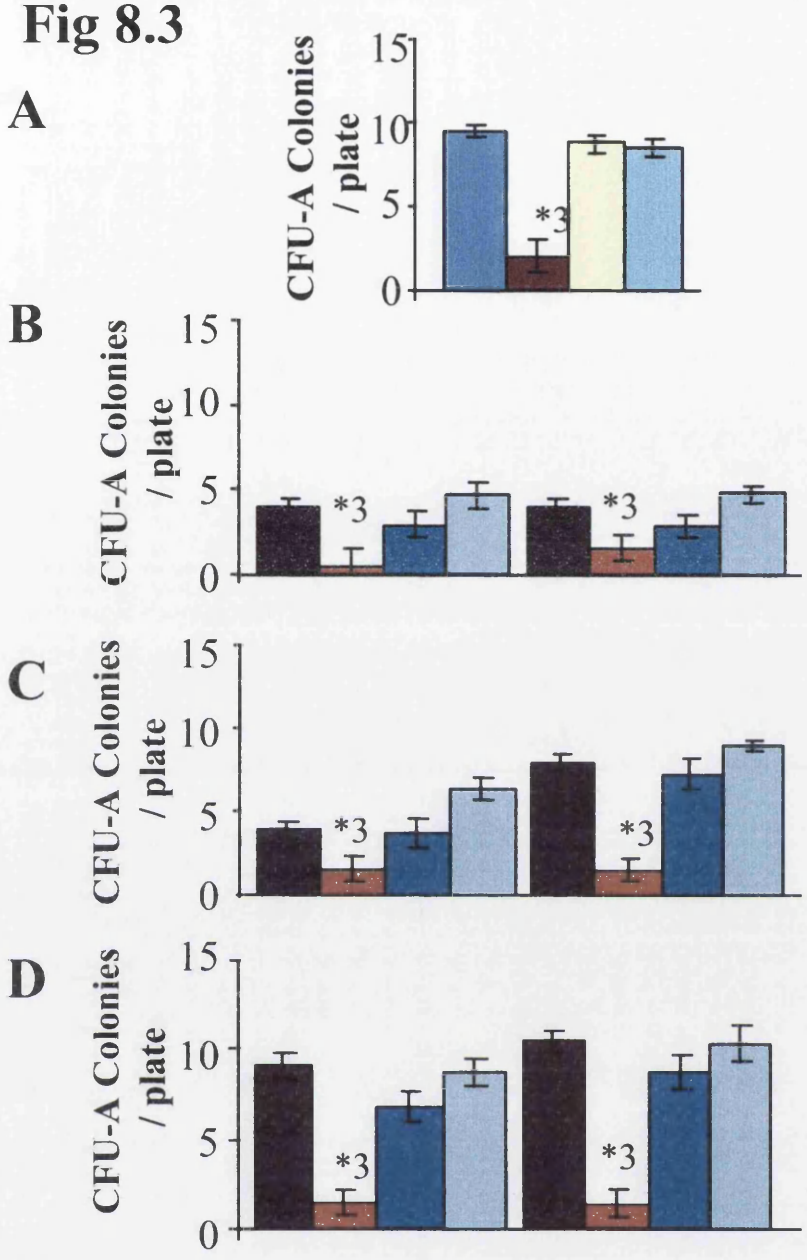


Figure 8.3 Colony Forming Unit Agar (CFU-A) analysis of the effect of Interleukin-11 at 0.5, 1, 5, 10, 50 and 100 ng/ml on the inhibitory effect of MIP-1α in a CFU-A assay

A CFU-A assay was set up that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF + MIP-1α at 1, 10 and 100 ng/ml. This assay was set up in the absence {A} or the presence of Interleukin-11 at 100 and 50 {B}, 10 and 5 {C}, 1 and 0.5 ng/ml {D}. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 100 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-11
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-11 + 100 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-11 + 10 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-11 + 1 ng/ml MIP-1α

Fig 8.4

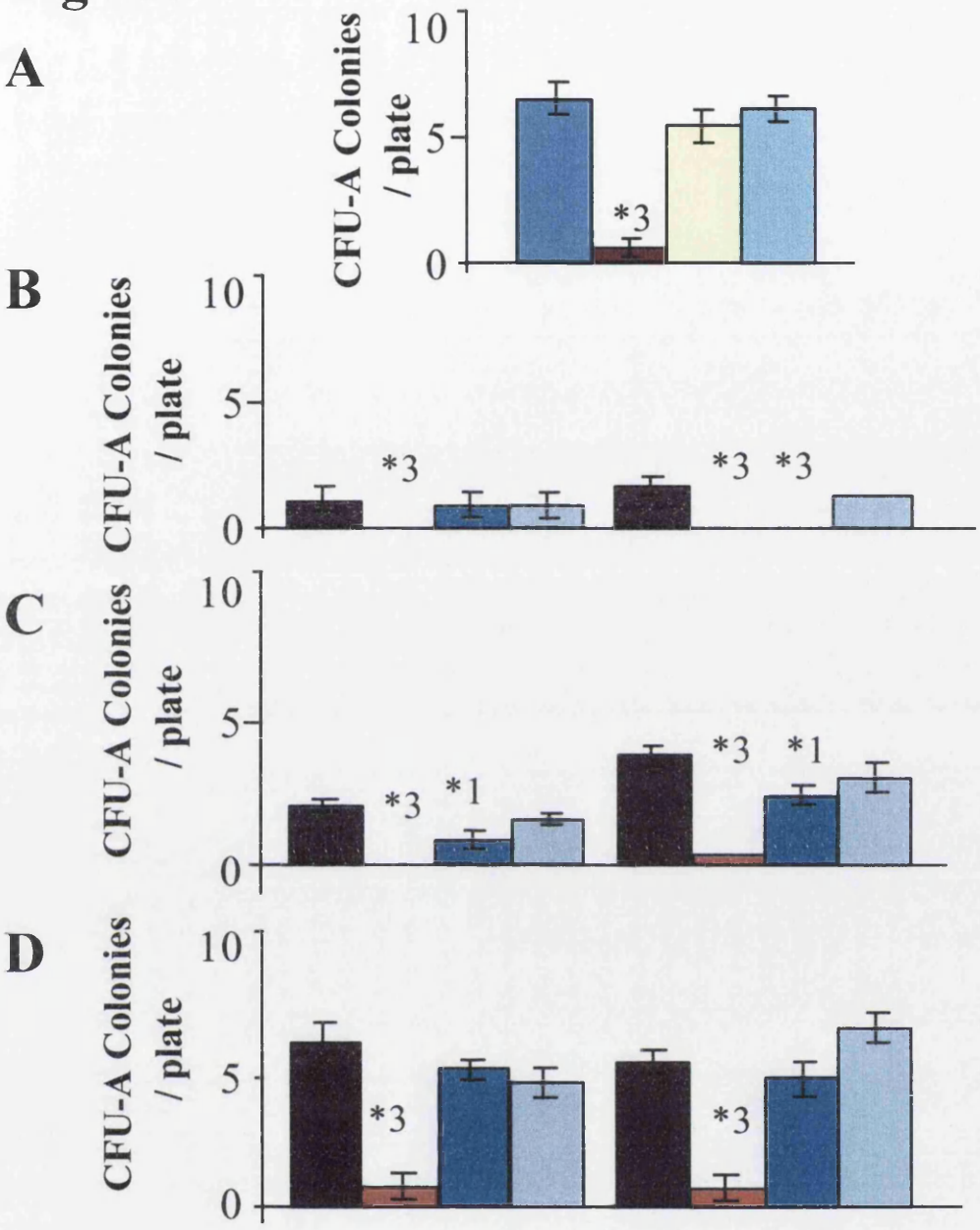


Figure 8.4 Colony Forming Unit Agar (CFU-A) analysis of the effect of Interleukin-6 at 0.5, 1, 5, 10, 50 and 100 ng/ml on the inhibitory effect of MIP-1α in a CFU-A assay

A CFU-A assay was set up that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF + MIP-1α at 1, 10 and 100 ng/ml. This assay was set up in the absence {A} or the presence of Interleukin-6 at 100 and 50 {B}, 10 and 5 {C}, 1 and 0.5 ng/ml {D}. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 100 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-6
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-6 + 100 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-6 + 10 ng/ml MIP-1α
- 100, 50, 10, 5, 1 and 0.5ng/ml IL-6 + 1 ng/ml MIP-1α

8.2.3 The Effect of LIF on CFU-A Colony Formation

In an attempt to see if the apparent reduction in CFU-A colonies was shared by all the gp130 signalling cytokines, a titration of Leukaemia Inhibitory Factor (LIF) in the presence or absence of rm MIP-1α at 1, 10 or 100 ng/ml was set up. Figure 8.5 and 8.6 both indicate that rh LIF has no effect on normal colony production or inhibition of CFU-A colonies when it is added to a CFU-A assay. McKinstry *et al* observed that isolated LTRC, STRC and progenitor cells from bone marrow can not bind radiolabelled LIF suggesting that LIF receptors are not found on these cells (Mckinstry *et al* 1997). This may explain why rh LIF has no effect on the formation or inhibition of CFU-A colonies. The inhibition of CFU-A stem cells by rh IL-11 and rh IL-6, is not a property that has been normally associated with either of these cytokines and thus, further analysis was performed to examine what is occurring in these assays.

8.2.4 The Effect of IL-11 and IL-6 on the Constituent Cells of the CFU-A Colonies

In order to examine the effects of rh IL-11 and rh IL-6 further, a CFU-A assay was set up to examine the effects on the number and types of cells in each CFU-A colony in the presence or absence of 100 ng/ml of MIP-1α (table 7 and 8). Upon the addition of 10 ng/ml and 100 ng/ml of rh IL-11, the number of cells within the CFU-A colonies are reduced from 20000 to 18800 and 16400 respectively. Upon the addition of 100 ng/ml of rm MIP-1α, it was observed that there was a further reduction in the number of cells within each colony to 7750 and 5800. In a control CFU-A assay, it can also be observed that the colonies predominantly consist of macrophages (approximately 98 %) with a small number of granulocytes, predominantly neutrophils, and blast cells. However, on the addition of 100 ng/ml of rh IL-11, there is an increase in the number of granulocytes observed in the CFU-A colonies from 0.4 to 4 % with a similar decrease in the number of macrophages. In similar assays containing rh IL-6, instead of rh IL-11, it can be seen that on the addition of 10 ng/ml and 100 ng/ml of rh IL-6 the number of cells within the CFU-A colonies are reduced from 20000 to 16400 and 10400 respectively. Furthermore, upon the addition of 100 ng/ml of rm MIP-1α

Fig 8.5

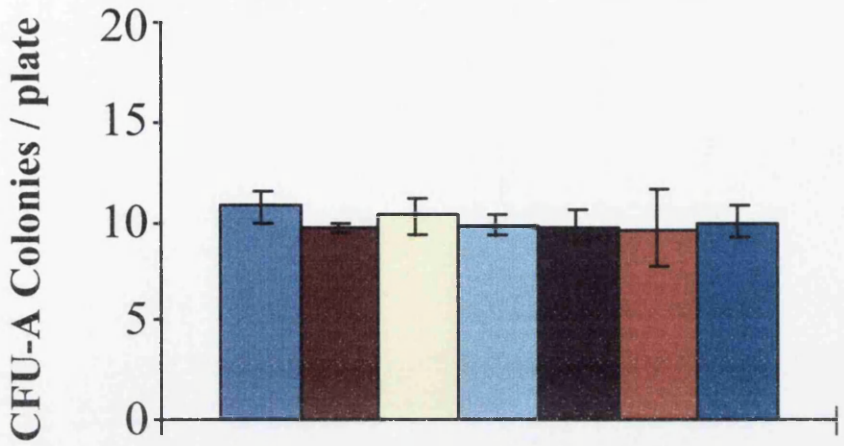


Figure 8.5 Colony Forming Unit Agar (CFU-A) analysis of the effect of Leukaemia Inhibitory Factor on the CFU-A colony production

Leukaemia Inhibitory Factor at 0.5, 1, 5, 10, 50 and 100 ng/ml was added to a normal CFU-A assay containing 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 100 ng/ml LIF
- 50 ng/ml LIF
- 10 ng/ml LIF
- 5 ng/ml LIF
- 1 ng/ml LIF
- 0.5 ng/ml LIF

Fig 8.6

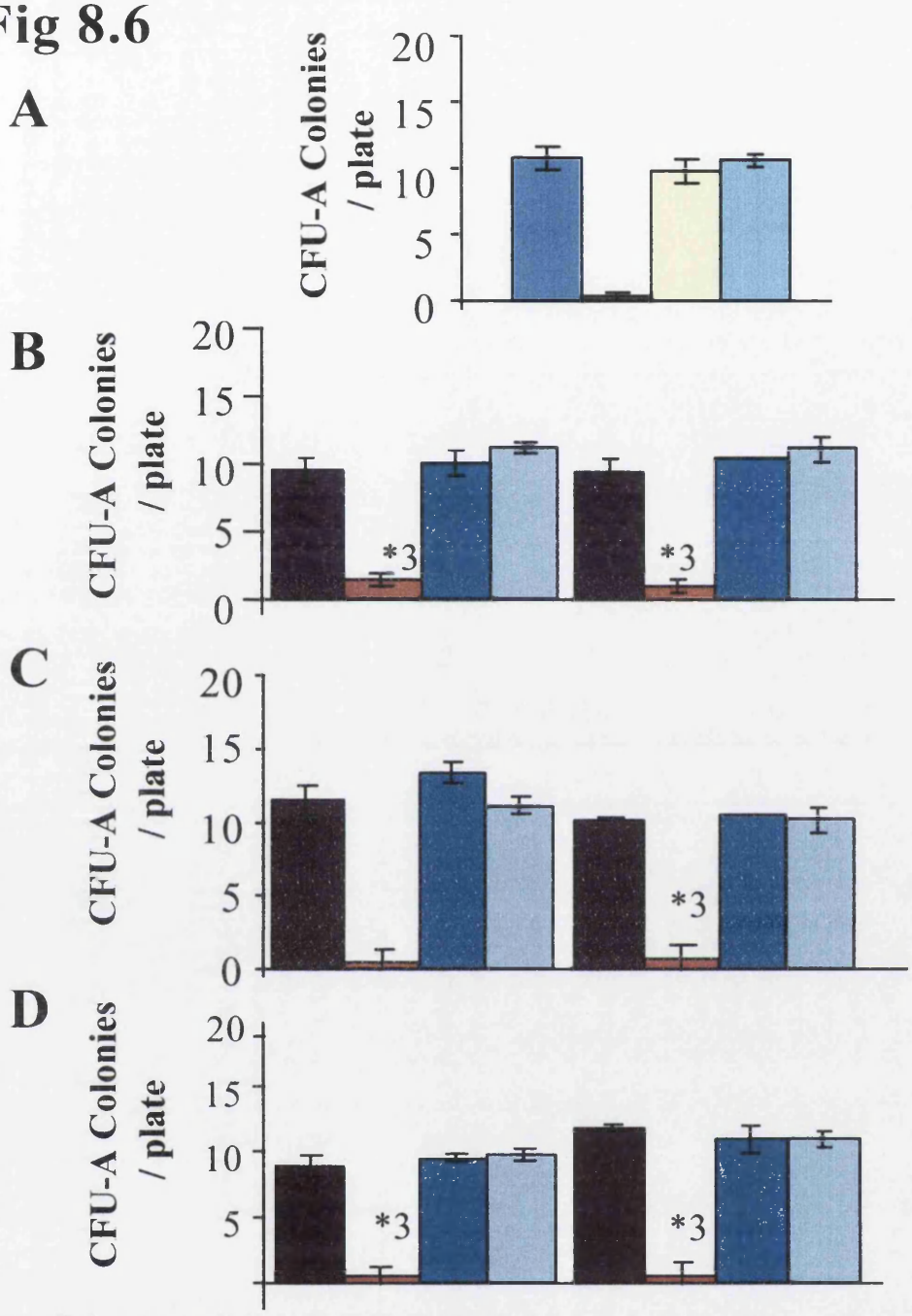


Figure 8.6 Colony Forming Unit Agar (CFU-A) analysis of the effect of LIF at 0.5, 1, 5, 10, 50 and 100 ng/ml on the inhibitory effect of MIP-1α in a CFU-A assay

A CFU-A assay was set up that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF + MIP-1α at 1, 10 and 100 ng/ml. This assay was set up in the absence {A} or the presence of LIF at 100 and 50 {B}, 10 and 5 {C}, 1 and 0.5 ng/ml {D}. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

Table 7

Cell Numbers within CFU-A colonies

Normal	20000
100 ng/ml MIP-1α	5500
rh IL-11 100 ng/ml	16400
rh IL-11 100 ng/ml + MIP-1α	5800
rh IL-11 10 ng/ml	18800
rh IL-11 10 ng/ml + MIP-1α	7750
rh IL-11 1 ng/ml	24500
rh IL-11 1 ng/ml + MIP-1α	7100

Differential counts (500 cells)

	Macrophages	Granulocytes	Others
Normal	498	2	0
100 ng/ml MIP-1α	490	5	5
rh IL-11 100 ng/ml	480	20	0
rh IL-11 100 ng/ml + MIP-1α	482	17	1
rh IL-11 10 ng/ml	492	4	4
rh IL-11 10 ng/ml + MIP-1α	495	5	0
rh IL-11 1 ng/ml	498	2	0
rh IL-11 1 ng/ml + MIP-1α	499	1	0

Table 7 Analysis of cell number and morphology of the cells within the CFU-A colonies containing 1, 10 or 100 ng/ml of Interleukin-11 in the presence or absence of 100 ng/ml MIP-1α

CFU-A assays were set up containing a top layer of methylcellulose instead of the normal double agar assay. 10 colonies were picked from plates containing 100 ng/ml MIP-1α and 5 colonies from the plates that did not contain MIP-1α. These cells were washed and either cytospins were prepared slides and stained by Giemsa for morphology analysis or cell numbers were counted.

Table 8

Cell Numbers within CFU-A colonies

Normal	20000
100 ng/ml MIP-1α	5500
rh IL-6 100 ng/ml	10400
rh IL-6 100 ng/ml + MIP-1α	6700
rh IL-6 10 ng/ml	16400
rh IL-6 10 ng/ml + MIP-1α	12550
rh IL-6 1 ng/ml	22500
rh IL-6 1 ng/ml + MIP-1α	9000

Differential counts (500 cells)

	Macrophages	Granulocytes	Others
Normal	498	2	0
100 ng/ml MIP-1α	490	5	5
rh IL-6 100 ng/ml	300	150	50
rh IL-6 100 ng/ml + MIP-1α	425	25	50
rh IL-6 10 ng/ml	390	9	1
rh IL-6 10 ng/ml + MIP-1α	370	30	0
rh IL-6 1 ng/ml	493	7	0
rh IL-6 1 ng/ml + MIP-1α	450	50	0

Table 8 Analysis of cell number and morphology of the cells within the CFU-A colonies containing 1, 10 and 100 ng/ml of Interleukin-6 in the presence or absence of 100 ng/ml MIP-1α

CFU-A assays were set up containing a top layer of methylcellulose instead of the normal double agar assay. 10 colonies were picked from plates containing 100 ng/ml MIP-1α and 5 colonies from the plates that did not contain MIP-1α. These cells were washed and either cytospins were prepared slides and stained by Giemsa for morphology analysis or cell numbers were counted.

it was observed that the number of cells was further reduced within each colony to 12550 and 6700. It can also be seen that with the inclusion of 100 ng/ml of rh IL-6 in the CFU-A assays, there is an increase in the number of granulocytes observed in the CFU-A colonies from 0.4 to 30 %, an increase in the number of blast cells from 0 to 10 % and a subsequent decrease in the number of macrophages from 99.6 % to 60 %. The alteration of the constituent cells of the CFU-A colonies upon the addition of rh IL-11 and rh IL-6 suggest that these cytokines are, in some way, interacting with the CFU-A stem cells and maybe altering their ability to differentiate along the pathways that normally lead to the formation of CFU-A colonies. The alteration in granulocytes with rhIL-11 and blast cell number with rhIL-6 has not been observed in any of the previous assays that were examining the alteration of growth factor concentrations. Blast cells are primitive haemopoietic cells and characteristically display a high nuclear to cytoplasmic ratio and this morphology is shared by stem cells. Therefore, as suggested from the data in table 8, it seems that rh IL-6 may in fact be increasing the number of blast cells in these colonies by increasing the level of the stem cell renewal with a concomitant decrease in differentiation. This would explain the alteration in the constituent cells of the CFU-A colony, and may also explain the apparent reduction in CFU-A colony numbers at high levels of hIL-6, see discussion. The replating ability of the CFU-A colonies that contained an increased number of blast cells was performed, however the data was inconclusive, data not shown.

8.3 Summary

In summary, the overall trend produced with the inclusion of rh IL-6 in the CFU-A assay is similar to the results with rh IL-11 in that both cytokines at the higher concentrations reduced/ inhibited the number of CFU-A colonies. At lower concentrations of both these cytokines, the reduction or inhibition in CFU-A numbers is not as clear. Unlike rh IL-11, rh IL-6 can enhance the ability of rm MIP-1α to reduce CFU-A colony formation as 10 ng/ml of rm MIP-1α was observed to induce a significant reduction in CFU-A colony number. This concentration of rm MIP-1α is not normally associated with the reduction in CFU-A colony formation, therefore, it seems that rh IL-6 can interact with rm MIP-1α and induce an additive reduction in CFU-A colony numbers. Alternatively the decrease in colony formation may be due to IL-6 and IL-11 acting upon the progeny of the CFU-A stem cell, e.g CFU-GM or CFU-G, in such a way that they stimulate the formation of neutrophils from these

progenitor cells. There are a number of reasons why this may occur and these are discussed later in section 10.3.1. Recombinant human IL-11 and rh IL-6 also have the ability to alter the constituent cells of the CFU-A colony and again IL-6 has a more prominent effect than IL-11. Interestingly, these abilities of rh IL-11 and rh IL-6 to affect the inhibition of CFU-A colonies and the constituent cells of the colonies is not a function that is shared by all gp130 signalling cytokines. Indeed, rh LIF does not share the activities of IL-11 or IL-6 observed above and this may be due to the lack of LIFRα sub-units expressed on the CFU-A stem cells.

8.4 Overall Summary of the Effects of CFU-A Growth Conditions on MIP-1α Inhibition of CFU-A Colony Formation

Overall, it can be observed in these studies that rm SCF, rh M-CSF and rm GM-CSF are essential for the formation of CFU-A colonies. An increase or reduction in the stimulus through the tyrosine kinase and the β common signalling pathways by rh M-CSF/rm SCF and rm GM-CSF respectively, appear to minimally interfere with the inhibition of CFU-A colony formation by rm MIP-1α. However, high concentrations of SCF and MCSF in the presence of low MIP-1α concentrations were observed to increase CFU-A colony numbers. Furthermore, an increase in concentration of rm GM-CSF in the presence of rm MIP-1α was observed to induce the formation of an alternative colony shape that may be induced through the activation of a signalling pathway through the chemokine receptor CCR-1. Upon the addition of gp130 signalling cytokines to the CFU-A assay, it was observed that rh IL-6 and rh IL-11 could reduce the number of CFU-A colonies produced and rh IL-6, but not rhIL-11, was observed to further reduce the colony number with the addition of rm MIP-1α. Recombinant human LIF had no effect on colony number or inhibition by MIP-1α and this maybe due to the level of receptor expression. The inability of rm SCF, rh M-CSF, rh IL-11 and rh LIF to interfere with rm MIP-1α induced inhibition may be due to the CFU-A assay being an inappropriate system to analyse the effect of MIP-1α on the inhibition of the proliferation of CFU-A stem cells. The CFU-A assay by definition is examining the differentiation of CFU-A stem cells into CFU-A colonies and therefore, it may be possible to interfere with the inhibitory effect of MIP-1α in a proliferation-based assay. Such a proliferation-based assay is described and examined in the chapter 9.

CHAPTER 9

RESULTS 5 : Ex-Vivo Expansion

9.1 Introduction

In the previous chapters, the alterations in the growth factor concentration were observed to have a limited ability to interfere with the inhibition of CFU-A colony formation by MIP-1α. This may be because there is a limited interaction between the growth promoting signalling and inhibitory signalling pathways, but it may also be due to the inappropriateness of the CFU-A assay. The CFU-A assay by definition examines the differentiation of CFU-A stem cells into CFU-A colonies. Therefore, it is possible that the role of rm SCF, rm GM-CSF and rh M-CSF is to prevent cell death, and upon doing so they allow the differentiation of the CFU-A stem cells and thus play a minimal role in the direct proliferation of the CFU-A stem cells. If this is the case, the effect of altering the growth factor concentrations on MIP-1α responses may be easier to see in a short trem proliferative assay e.g ex-vivo expansion assay..

Cytokine treatment of murine bone marrow has been observed to be an alternative source of transiently engrafting haemopoietic stem cells but not primitive haemopoietic stem cells for transplantation after myeloablative therapy (Peters *et al* 1996, Varas *et al* 1998). The characterisation of numerous cytokines involved in the control of haemopoiesis and, their potential use in *ex-vivo* cultures to expand stem and progenitor cells has established these growth factors not only as candidates for use in expanding cells prior to transplantation but also for gene therapy (Bernard *et al* 1994). In *ex-vivo* culture systems, combinations of synergistic growth factors are needed to provide signals that allow the stem cells to survive and proliferate. IL-3 and SCF have been observed to act as survival factors for dormant murine haemopoietic progenitor and stem cells (Katayama *et al* 1993, Bodine *et al* 1989). As well as acting as a survival factor, SCF in combination with IL-6, IL-11 and G-CSF has the ability to shorten the time that these cells spend in Go (Tsuji *et al* 1991). Prior to the cloning of SCF, the most potent combination of synergising growth factors was IL-3 and IL-6 (Bodine *et al* 1989) however, with the identification and inclusion of SCF in these expansion protocols an even greater expansion of stem and progenitor cells could be produced (Bodine *et al* 1992). Flt 3 ligand, signals through its receptor Flk 2, which

is a member of the tyrosine kinase family, is another early acting cytokine (Zeigler *et al* 1994). Similar to SCF, Flt 3 has been observed to produce strong synergy when combined with ligands that signal through the hematopoietin family of receptors (G-CSF, GM-CSF, IL-3, IL-6, IL-11 and IL-12) (Haylock *et al* 1997, Kobari *et al* 1998). A less potent synergy was found when Flt3 was used in combination with growth factors that signal through other members of the tyrosine kinase family, eg SCF or M-CSF. Therefore, the activation of a tyrosine kinase receptor pathway along with the activation of a hematopoietin receptor pathway, by their several ligands, produces a larger expansion of stem and progenitor cells compared to the activation of two similar receptor signalling pathways.

9.2 Ex-vivo Expansion

9.2.1 Expansion of CFU-A Stem Cells

Holyoake *et al* previously optimised the *ex-vivo* culture conditions that allowed the amplification of murine transiently engrafting stem cells, as measured by the CFU-A assay (Holyoake *et al* 1996). Using a variety of single growth factors or a combination of growth factors, they observed that the most impressive amplification was consistently obtained with rm SCF and rh IL-11 at 18 and 100 ng/ml respectively. To confirm this a number of expansion experiments were performed, 2 x 10^5 unfractionated murine bone marrow cells were cultured in α-MEM supplemented with 25 % DHS and grown in 35 mm petri dishes for 6 days in the presence of various single growth factors or combinations of these growth factors. These *ex-vivo* expansion experiments have been repeated several times and the results of one is outlined in figures 9.1 and 9.2. CFU-A colony production was enumerated before and after the expansion and the amplification factor was derived using this equation:

$$\frac{\text{Number of cells of normal bone marrow plated}}{\text{Number of cells of expanded bone marrow plated}} \times \frac{\text{Number of CFU-A colonies in expanded bone marrow}}{\text{Number of CFU-A colonies in normal bone marrow}}$$

Fig 9.1

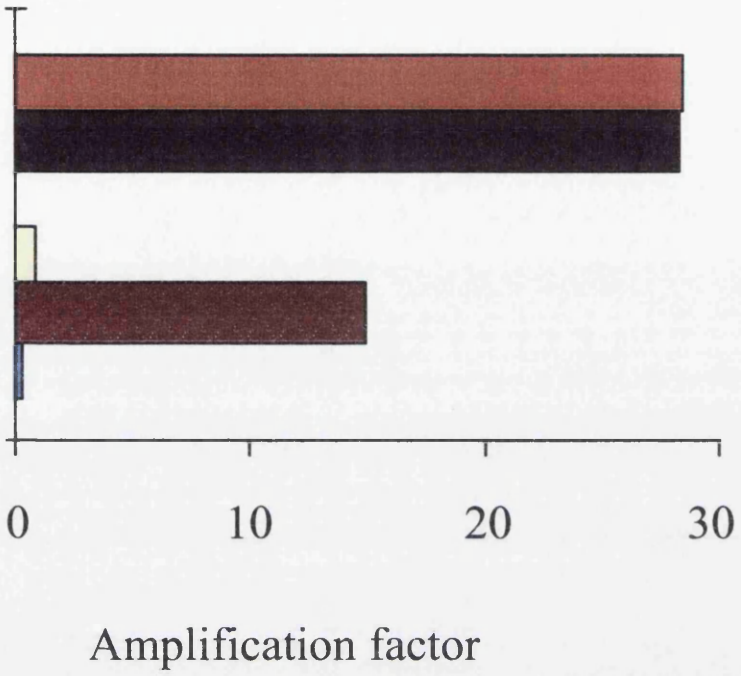


Figure 9.1 Colony Forming Unit Analysis of the expansion of CFU-A stem cells by various single growth factors and combinations of growth factors

Unfractionated bone marrow was cultured for 6 days in α-MEM media. Cytokines were added either singly or in combination at the start of the culture. mSCF at 18 ng/ml, rhIL-11 100 ng/ml, rmMIP-1α 100 ng/ml, mSCF and rhIL-11 or rm SCF, rh IL-11 and rmMIP-1α. Unfractionated bone marrow or expanded cells were plated in CFU-A assay that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. The amplification factor is shown for each treatment. This assay is a representative result from five replicate experiments.

- Medium
- SCF 18 ng/ml
- IL-11 100 ng/ml
- MIP-1α 100 ng/ml
- SCF 18 ng/ml and IL-11 100 ng/ml
- SCF 18 ng/ml, IL-11 100 ng/ml and 100 ng/ml MIP-1α

Fig 9.2

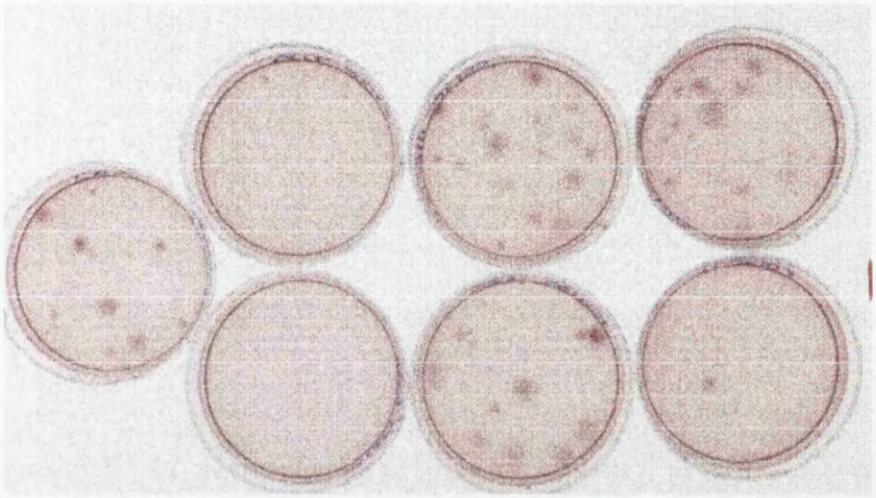
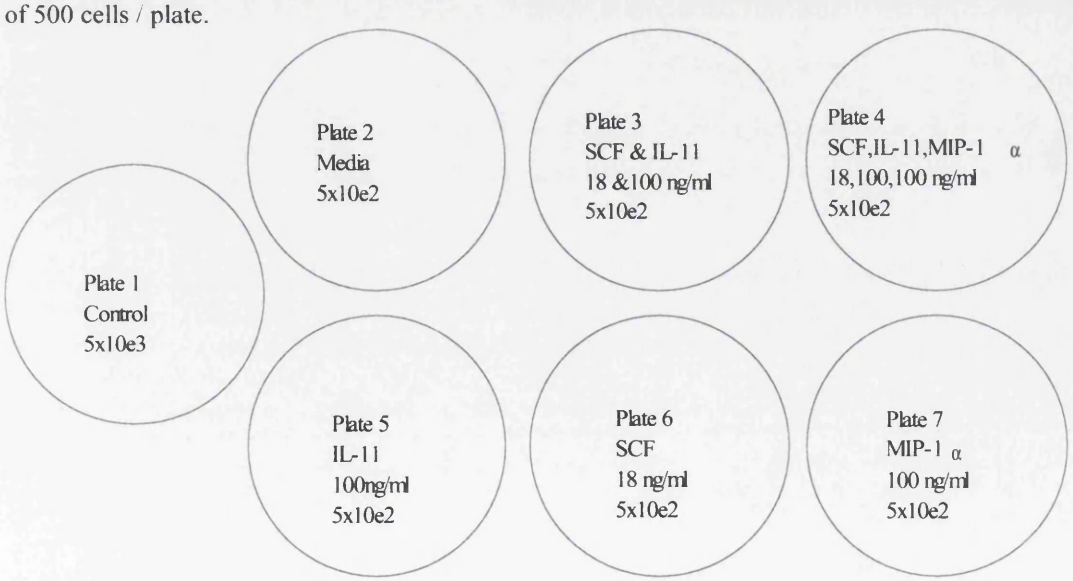


Figure 9.2 Morphological analysis of expanded CFU-A colonies by photography

Photographs of CFU-A colonies were produced as described in materials and methods. The CFU-A colonies were stained with INT and incubated overnight. This assay contains the normal concentrations of the three basic growth factors within the CFU-A assay 12 ng/ml rm SCF, 6 ng/ml rh M-CSF and 0.2 ng/ml rm GM-CSF.

The plate 1 is the control CFU-A assay containing unfractionated non expanded cells plated at a final concentration of 5000 cells / plate.
In the top row plate 2 contains cells grown for 6 days in media alone and plated at a final concentration of 500 cells / plate.
The plate 3 contains cells grown for 6 days in 18 ng/ml of mSCF and 100 ng/ml rhIL-11and plated at a final concentration of 500 cells / plate.
The plate 4 contains cells grown for 6 days in 18 ng/ml of rm SCF and 100 ng/ml of rh IL-11 and 100 ng/ml MIP-1α and plated at a final concentration of 500 cells / plate.
In the lower row the plate 5contains cells grown for 6 days in 100 ng/ml rh IL-11 plated at a final concentration of 500 cells / plate.
The plate 6 contains cells grown for 6 days in 18 ng/ml of rm SCF and plated at a final concentration of 500 cells / plate.
The plate 7 contains cells grown for 6 days in 100 ng/ml of MIP-1α and plated at a final concentration of 500 cells / plate.



The incubation of unfractionated bone marrow in α-MEM alone or with rh IL-11 or rm MIP-1α could not produce any significant amplification of CFU-A stem cell numbers. The only single growth factor to produce an amplification of CFU-A numbers was rm SCF; however, the amplification was never as great as that observed with the combination of SCF and IL-11. The effect of the addition of rm MIP-1α to the expansion of CFU-A stem cells numbers by rm SCF and rh IL-11 was examined next. MIP-1α has been shown to have bi-directional effects (i.e. stimulatory or inhibitory) depending on the maturation stage of the target population, this is also the case for TGF-β. The further addition of 100 ng/ml of rm MIP-1α a concentration known to inhibit CFU-A colony production in soft agar assays, to the expansion cocktail of rm SCF and rh IL-11 produced no positive or negative effects on the amplification of CFU-A numbers (figure 9.1). This result indicated that the synergistic activity of rm SCF and rh IL-11 could somehow interfere with the inhibitory activity of MIP-1α. Alternatively it is possible that the action of SCF or IL-11 on the constituent cells of the bone marrow induce the production of another factor/s that interfere with the MIP-1α inhibition of CFU-A colony formation. The prepartion of conditioned media form the expanded bone marrow and the examination of its effect to interfere with MIP-1α inhibtion of CFU-A colony formation would be an ideal experiment to analyse this effect, however due to time restraints this was not examined.

9.2.2 The Effects of Various Sources of SCF on the Expansion of CFU-A Stem Cells

The SCF used in these expansion experiments was in medium conditioned by CHO cells expressing soluble kit ligand (Anderson *et al* 1990). Due to the possibility that unknown factors within the conditioned media could be producing this expansion by synergising with SCF, the expansion of CFU-A stem cells using recombinant human and murine SCF + rh IL-11 was compared to the combination of CM SCF and IL-11. In figure 9.3, it can be observed that human SCF in combination with IL-11 produced a small amplification of CFU-A numbers, whereas rm SCF/IL-11 and CM SCF/IL-11 could produce an approximately 40-fold amplification in CFU-A stem cells. This inability of cross species activity has been observed before for SCF. These results suggest that the impurities that may exist within the CM do not enhance the expansion of CFU-A stem cells by SCF and IL-11, in fact they may slightly reduce it. Therefore,

Fig 9.3

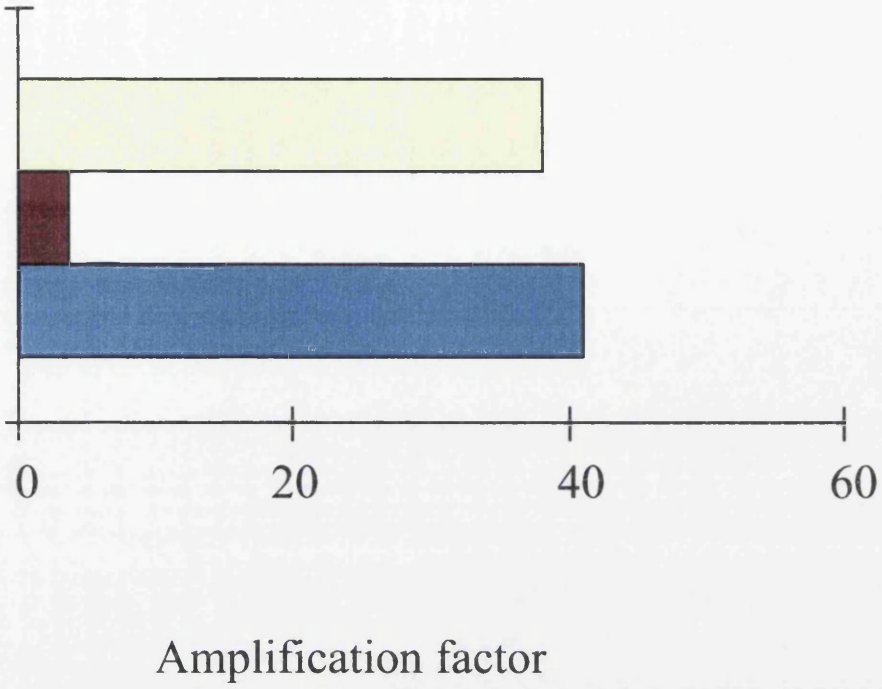


Figure 9.3 Colony Forming Unit Agar analysis of the expansion of CFU-A stem cells rh IL-11 and various sources of SCF

Unfractionated bone marrow was cultured for 6 days in α-MEM media. Combinations of rmSCF 18 ng/ml +IL-11 100 ng/ml, rhSCF 18 ng/ml +IL-11 100 ng/ml and conditioned media SCF 18 ng/ml +IL-11 100 ng/ml were added at the start of the culture. Expanded cells were plated in CFU-A assay that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. Conditioned media is from CHO cells (Anderson *et al* 1990). The amplification factor is shown for each treatment. This assay is a representative result from five replicate experiments.

- rm SCF + IL-11
- rh SCF + IL-11
- cm SCF + IL-11

it seems that the amplification in CFU-A stem cells observed in these experiment is likely to be due the synergism between SCF and IL-11, (figure 9.3).

9.2.3 The Effect of MIP-1α on the Expansion of CFU-A Stem Cells

The ability of the synergistic combination of SCF and IL-11 to interfere with the MIP-1α induced inhibitory pathway was further analysed in figure 9.4. A CFU-A assay was set up to examine the effects of a dose response of MIP-1α on the inhibition of formation of CFU-A colonies from normal bone marrow and expanded bone marrow. Bone marrow cells were incubated for 6 days with SCF and IL-11, the expanded cells were then harvested, washed and plated in a CFU-A assay in the presence of 0.5, 1, 5, 10, 50 and 100 ng/ml of MIP-1α and compared to a CFU-A assay using non expanded bone marrow. From the results in figure 9.4, it can be observed that only MIP-1α at 100 and 50 ng/ml can inhibit the formation of CFU-A colonies from normal bone marrow. However, on the analysis of the inhibition of CFU-A colony formation by MIP-1α on expanded bone marrow cells, it can be observed that 50 ng/ml of MIP-1α can not induce inhibition of CFU-A colony formation. Also MIP-1α at 100 ng/ml can only reduce the formation of CFU-A colonies by 70 % compared to that of 100 % in the normal CFU-A assay. These results further indicate that the expansion of bone marrow with rm SCF and rh IL-11 can somehow interfere with the inhibitory property of rm MIP-1α. Interestingly, the morphology of the CFU-A colonies produced from the expanded bone marrow in CFU-A assays containing 0.5, 1, 5, 10 and 50 ng/ml of MIP-1α were similar to the alternative colonies described in section 7.2.3.

9.2.4 The Effect of TGF-β on the Expanded CFU-A Stem Cells

MIP-1α is not the only molecule that has been observed to inhibit the formation of CFU-A colonies, TGF-β has also been observed to possess inhibitory properties with respect to CFU-A colony formation. Therefore, a further experiment was set up to see if the expansion of bone marrow by rm SCF and rh IL-11 could render the CFU-A stem cells unresponsive towards TGF-β. Bone marrow cells were expanded, as previously described, and 5000 normal bone marrow cells/plate or 500 expanded bone marrow cells /plate were grown in the CFU-A assay in the presence and absence of either MIP-1α or TGF-β at 100 ng/ml. From figure 9.5, it can be observed that both

Fig 9.4

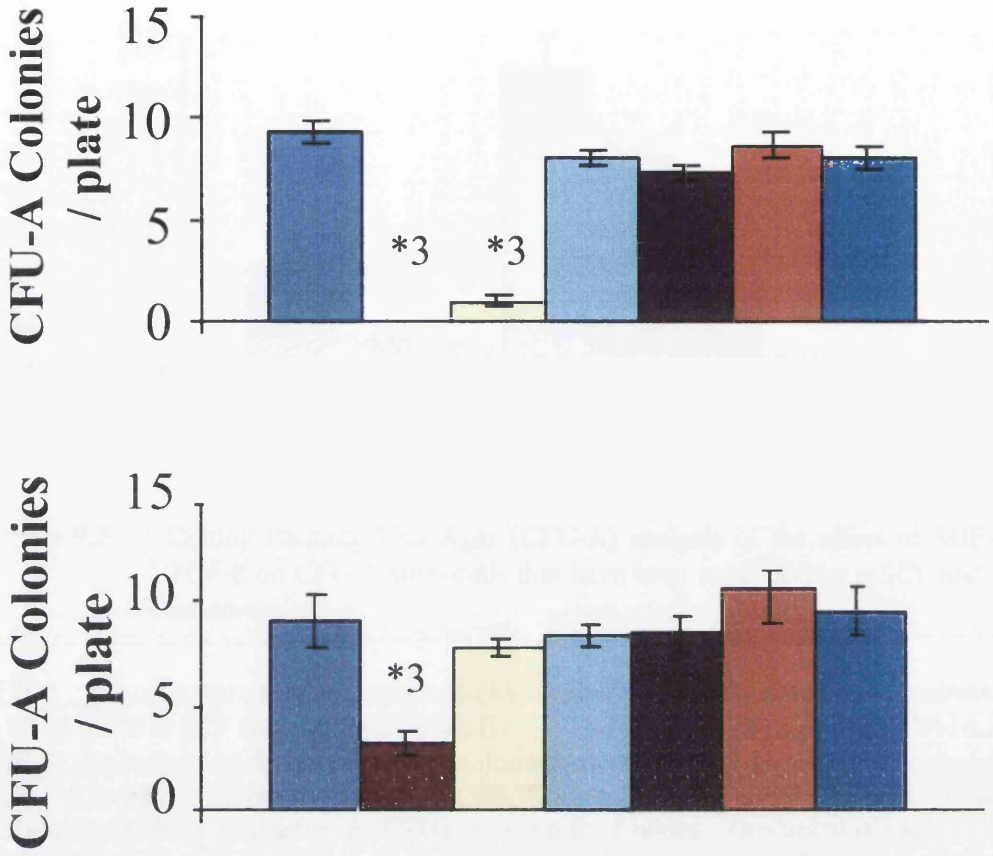


Figure 9.4 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α on the CFU-A colony production of normal and expanded bone marrow

Bone marrow was expanded for 6 days by incubating with 18 ng/ml of rmSCF and rh100 ng/ml of IL-11, washed and counted. 5000 normal bone marrow cells and 500 expanded bone marrow cells were plated in a CFU-A assay containing MIP-1α at 0.5, 1, 5, 10, 50 and 100 ng/ml and rm GM-CSF (0.2 ng/ml), rh M-CSF (6 ng/ml) and 12 ng/ml of rm SCF and these are represented in A and B respectively. The average colony production ($\pm$ SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing control (normal bone marrow) to all the other plates containing the various MIP-1α concentrations (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$).

- Control
- 100 ng/ml MIP-1α
- 50 ng/ml MIP-1α
- 10 ng/ml MIP-1α
- 5 ng/ml MIP-1α
- 1 ng/ml MIP-1α
- 0.5 ng/ml MIP-1α

Fig 9.5

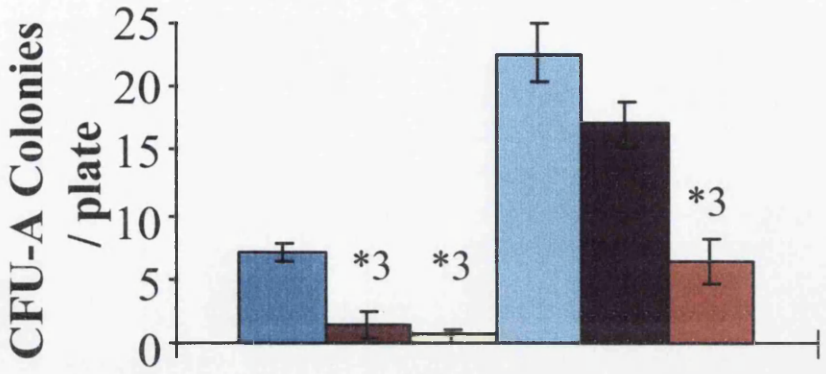


Figure 9.5 Colony Forming Unit Agar (CFU-A) analysis of the effect of MIP-1α and TGF-β on CFU-A stem cells that have been expanded by mSCF and rhIL-11 treatment.

CFU-A stem cells were routinely expanded by incubating unfractionated bone marrow cells in 18 ng/ml of m SCF and 100 ng/ml of rh IL-11. The effect of 100 ng/ml of MIP-1α and TGF-β (two known inhibitors of CFU-A colonies) on the CFU-A formation was examined in a CFU-A assay containing 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. The average colony production (± SEM) is shown for 5 plates. This assay is a representative result from three replicate experiments. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 100 ng/ml MIP-1α
- 100 ng/ml TGF-β
- Expanded bone marrow
- Expanded cells 100 ng/ml MIP-1α
- Expanded cells 100 ng/ml TGF-β

MIP-1α and TGF-β can inhibit the formation of normal CFU-A colonies equally ($p < 0.001$). However, MIP-1α was not as effective at inhibiting the formation of CFU-A colonies in assays using expanded bone marrow cells. MIP-1α could only inhibit 20 % of the CFU-A colony formation in assays using expanded bone marrow compared to that of 90 % in assays using normal bone marrow. TGF-β can however, inhibit the formation of CFU-A colonies from expanded stem cells. From these results, TGF-β can be observed to inhibit the formation of CFU-A colonies in both normal and expanded bone marrow therefore, the expansion of stem cells by rm SCF and rh IL-11 in some way is specifically interfering with the inhibitory action of MIP-1α.

9.2.5 Receptor Expression of Lineage Depleted Normal and Expanded Bone Marrow Cells

It was hypothesised that this inability of MIP-1α to inhibit CFU-A colony formation after the expansion of CFU-A stem cells by rm SCF and rh IL-11 may be due to down regulation of the MIP-1α inhibitory receptor. Therefore, the expression of messenger RNA for the various known MIP-1α receptors was examined in a lineage-depleted population of normal and expanded bone marrow. Lineage depletion was performed on normal and expanded bone marrow cells in an attempt to produce a more comparable cellular population. Bone marrow and expanded CFU-A stem cells were firstly depleted of cells that expressed the mature lineage markers B220, Gr-1, Ter119, CD4, CD5 and CD8, and then total RNA was isolated from these lineage negative cells. 20 μg of total RNA from both cell samples were separated on an agarose gel and then immobilised on Hybond N membranes. These RNA populations were then examined for the expression of four CC chemokine receptors namely CCR-1, CCR-3, CCR-5 and D6. The message for CCR-3 and D6 receptors could not be detected by this method however, as observed in figure 9.6, both CCR-1 and 5 could be detected in both lineage depleted populations, the expression of β-actin, the house keeping gene, was used as a loading control. Two bands of 2.8 kb and 3.7 kb were detected when both populations were probed for CCR-5 expression and these sizes compare favourably to that of 3.1 and 4 kb that was initially reported by Meyer *et al* (Meyer *et al* 1996). The results from figure 9.6, indicate that CCR-5 levels are unaltered in the expanded bone marrow lineage negative population.

Fig 9.6

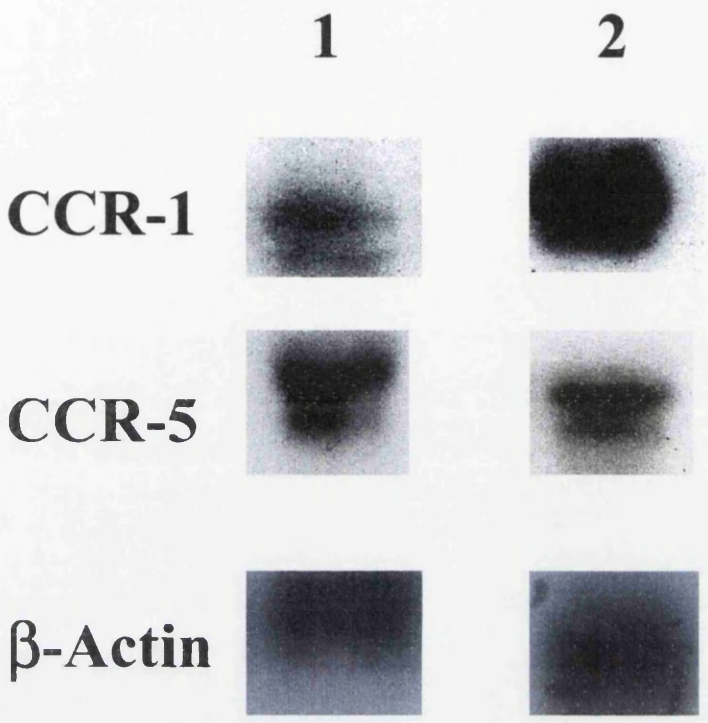


Figure 9.6 Northern blot analysis of the expression of chemokine receptors CCR-1, 5 and D6 after the expansion of CFU-A stem cells.

Bone marrow and expanded cells were firstly enriched for lineage negative populations. This was to ensure that both populations were similar to each other in cell types. 20 μg of total RNA from each lin$^-$ population was immobilised on each blot. The expression of CCR-1, 5 and D6 along with β-actin was examined; CCR3 and D6 could not be detected. This blot was stripped and reprobed after each hybridisation and the levels of loading were checked via ethidium bromide staining on the gel.

Column 1 shows RNA from lin$^-$ enriched bone marrow cells
Column 2 shows RNA from lin$^-$ enriched expanded cells

However, on the examination of CCR-1 receptor expression, it was observed that the levels of its mRNA were slightly increased in the expanded stem cells compared to the lineage depleted bone marrow cells. Two bands of 3.0 and 2.4 kb were observed after the blot was stripped and reprobed for CCR-1 expression. Gao *et al* identified three different specific mRNA bands in mouse leukocytes, which corresponded to mCCR-1, the major band was 2.4 kb, whereas the two minor bands were 3.7 and 6.0 kb. They suggested that the major 2.4 kb band appears as a broad band and therefore, it maybe made up of a collection of mRNA species (Gao *et al* 1995). The broad band may explain the isolation of the 3.0 kb band in figure 9.6, and the inability to detect the other minor bands maybe due to the isolation of RNA from different cellular populations. As described earlier in section 7.2.3, CFU-A colonies produced from expanded bone marrow and treated with rm MIP-1α were observed to display the alternative shape that was initially identified with high levels of rm GM-CSF. The formation of these alternative colonies and the increase in CCR-1 expression levels upon expansion of the bone marrow provides further evidence that CCR-1 may be involved in the formation of these alternatively shaped CFU-A colonies.

9.2.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis of CCR-3 and D6 Expression in Lineage Depleted Normal and Expanded Bone Marrow Cells

As indicated above northern blot analysis failed to reveal detectable expression of mRNA for the MIP-1α receptors CCR-3 and D6. The highly sensitive technique of reverse transcriptase polymerase chain reaction (RT-PCR) was therefore, used in an attempt to examine the expression of these receptors in lineage depleted bone marrow and expanded bone marrow. 1 μg of total RNA from each sample was used and the results are shown in figure 9.7, β-actin was used as a positive control and can be detected in both populations as a 247 bp band. The CCR-3 and D6 primers (section 3.13) used in the PCR reaction define fragments of 280 and 380 base pairs respectively. Although, the β-actin controls are uneven, it does indicate that CCR-3 and D6 can only be detected in the lineage depleted RNA from bone marrow and not in the lineage depleted RNA from the expanded stem cells, (figure 9.7). These results suggest that if the down regulation of the protein levels of D6 and CCR-3 are similar

Fig 9.7

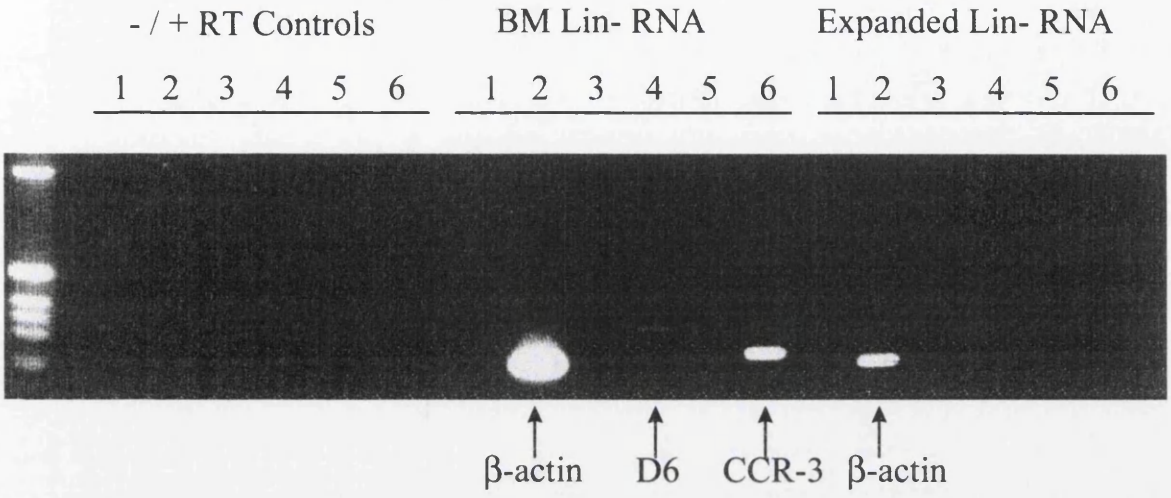


Figure 9.7 RT-PCR analysis of the expression of chemokine receptors CCR- 3 and D6 after the expansion of CFU-A stem cells

A master mix was made and split into + RT and - RT and reverse transcribed. A further PCR master mix was made up and divided up equally before the addition of + RT, -RT, no RNA or the different RNA samples and this was amplified up via 30 cycles of PCR as described in the materials and methods. 1μg of RNA was used from lineage enriched bone marrow and expanded cells. The primers that were used in the PCR reaction for CCR-3 and D6 will recognise bands of 280bp and 380bp respectively.

+/- RT	**Bone Marrow**	**Expanded Cells**
Lane 1 β-actin no RNA	β-actin no RNA	β-actin no RNA
Lane 2 β-actin RNA	β-actin RNA	β-actin RNA
Lane 3 D6 no RNA	D6 no RNA	D6 no RNA
Lane 4 D6 RNA	D6 RNA	D6 RNA
Lane 5 CCR-3 no RNA	CCR-3 no RNA	CCR-3 no RNA
Lane 6 CCR-3 RNA	CCR-3 RNA	CCR-3 RNA

to the RNA levels that either of these receptors may be the MIP-1α inhibitory receptor (see section 9.2.7 and in the discussion).

9.2.7 CCR-3 and D6 as possible Candidates for the Inhibitory Receptor

Both D6 and CCR-3 were down regulated when CFU-A stem cells are expanded and, as these expanded stem cells are unresponsive to MIP-1α it may be that either D6 or CCR-3 are in fact the inhibitory receptor. Recent work within our group comparing the inhibition of CFU-A colony formation using normal and D6 null mice bone marrow by MIP-1α, indicated that D6 is not the receptor that is responsible for the inhibitory activity of MIP-1α. As CCR3 null mice have yet to be made available, the analysis of the effect of MIP-1α on CFU-A colony formation from CCR3 bone marrow could not be performed. Therefore, a further experiment was set up to examine the role of CCR3 in MIP-1α inhibition of CFU-A colony formation. Murine Eotaxin does not inhibit CFU-A colony formation, Ottersbach personal communication, and displays a higher affinity for CCR3 than mMIP-1α, Nibbs personal communication. Therefore in an assay containing both Eotaxin and MIP-1α, Eotaxin will prevent MIP-1α from binding to CCR-3 and thus producing an inhibitory signal, that is if CCR-3 is in fact the inhibitory receptor used by MIP-1α. A CFU-A assay was performed containing a ten fold higher concentration of eotaxin (1μg/ml) than MIP-1α (100 ng/ml). Eotaxin at 1μg/ml could not inhibit CFU-A colony formation and MIP-1α alone or in the presence of 1μg/ml of Eotaxin could still inhibit CFU-A colony formation (figure 9.8). Therefore, these experiments indicate that MIP-1α must be inducing its inhibitory activity through a receptor other than CCR-3.

9.2.8 Summary

In summary, the expansion of bone marrow cells with rm SCF and rh IL-11 interferes with the ability of rm MIP-1α, but not rm TGF-β, to inhibit the proliferation and formation of CFU-A colonies. On analysis of MIP-1α receptor expression on rm SCF and rh IL-11 expanded stem cells it was also observed that CCR-3 and D6 receptor

Fig 9.8

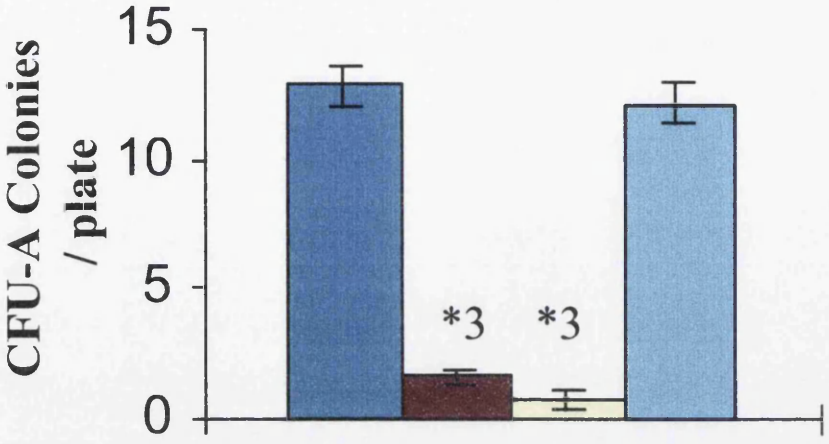


Figure 9.8 Colony Forming Unit Agar (CFU-A) analysis of the effect of Eotaxin and MIP-1α on the formation of CFU-A colonies

A CFU-A assay was set up that contained 6 ng/ml rh M-CSF, 12 ng/ml rm SCF and 0.2 ng/ml rm GM-CSF. To this was added either nothing, 100ng/ml of MIP-1α, 1μg/ml Eotaxin or 100ng/ml of MIP-1α and 1μg/ml Eotaxin. The average colony production (± SEM) is shown for 5 plates. This is a single experiment. Students T-test was performed comparing controls to all the treatments (*1 = $P < 0.05$, *2 = $P < 0.01$ and *3 = $P < 0.001$)

- Control
- 100 ng/ml MIP-1α
- 100 ng/ml MIP-1α + 1μg/ml Eotaxin
- 1μg/ml Eotaxin

levels were down regulated whereas, CCR-5 levels were unchanged and CCR-1 is up regulated. These results indicate that the expansion of CFU-A stem cells by rm SCF and rh IL-11 not only renders the CFU-A stem cells unresponsive to the inhibitory effect of MIP-1α, it also down regulates the expression of CCR-3 and D6 and up regulates the expression of CCR-1. These effects produced by expanding bone marrow by cytokine treatment may therefore, alter the response of the cells within the expanded population to the various chemokines.

10.0 DISCUSSION

10.1 Introduction

Our group is interested in chemokines, especially MIP-1α and its role as a haemopoietic stem cell inhibitor. MIP-1α was originally purified and characterised as a stem cell inhibitor, within our group, and further studies have indicated possible mechanisms for regulating the levels of MIP-1α *in-vivo* (Graham *et al* 1990, Maltman *et al* 1993 and 1996, Jarmin *et al* 1999). Although there has been a rapid expansion of the chemokine field in recent years, including the identification of various MIP-1α receptors, this has not enhanced our understanding of how MIP-1α induces the inhibition of CFU-A stem cell proliferation. Therefore, in this thesis I have attempted to examine how MIP-1α exerts its inhibitory activity at the molecular level, and whether altering the CFU-A growth factor concentrations could interfere with the MIP-1α induced inhibition of CFU-A colony formation.

From the results described in chapter 5, it can be concluded that the subtractive hybridisation was unsuccessful. Indeed, the two stem cell lines that were examined were deemed inappropriate for the study of MIP-1α inhibition, and the analysis of the cDNA isolated from the subtractive hybridisation indicated that the subtraction had not efficiently removed common genes. This therefore led to a change of direction aimed at examining if it was possible to interfere with MIP-1α inhibition of CFU-A colony formation by altering the CFU-A growth conditions and to attempt to infer from these results, modes of actions for MIP-1α.

10.2 CFU-A Growth Factor Alteration

10.2.1 The Effect of Omission of SCF on CFU-A Colony Formation

Before investigating the effect of growth factor concentration on MIP-1α inhibition of CFU-A colony formation, it was deemed necessary to firstly examine the effect of growth factor concentration on the formation of CFU-A colonies themselves. In the first experiment, the omission of SCF from the CFU-A assay was observed to produce a low level of CFU-A colony formation, compared to that of assays containing normal

levels of SCF (figure 6.3). This result confirmed the previous observation by Pragnell *et al*, who observed that, as well as GM-CSF and M-CSF, SCF is needed for optimal CFU-A colony growth (Pragnell *et al* 1994). Other studies examining the colony formation of human or murine haemopoietic stem and progenitor cells, have reported that SCF on its own has limited ability to induce colony formation. However, in combination with various growth factors SCF has been reported to increase the number and size of progenitor colonies (Broxmeyer *et al* 1991a, b, McNiece *et al* 1991a, b). These studies suggested that SCF enhances the proliferative response of progenitor cells to the other growth factors in the assays. Therefore, if this were the role that SCF plays within the CFU-A assay, this would explain the reduction in colony formation observed in figure 6.3 upon the omission of SCF. It is also possible that another function of SCF may explain the reduction in colony formation observed in figure 6.3. SCF has been observed to act as a survival factor for various cell types such as NK cells, mast cells, myelomonocytic cell lines and both human and murine stem and progenitor cells (Carson *et al* 1994, Gommerman and Berger 1998, Borge *et al* 1997, Li *et al* 1994). Therefore, it may be that within the context of the CFU-A assay, SCF functions to promote the survival of the CFU-A stem cells. However, as a small number of CFU-A colonies are produced in the absence of SCF, it may be that SCF is not acting as a survival factor and its main activity may be to enhance the proliferative response to other growth factors within the CFU-A assay. Although it is possible that GM-CSF or M-CSF may induce CFU-A stem cell survival in the absence of SCF, it is also feasible that the colonies observed in the CFU-A assays, in the absence of SCF, may have arisen from the increased growth of progenitor cells. Indeed, McNiece *et al* reported that M-CSF promoted the proliferation of a subset of HPP-CFC progenitors known as HPP-CFC-3 cells (McNiece *et al* 1988). Therefore, it may be that the interaction of M-CSF and GM-CSF in these CFU-A assays induces the growth of these colonies, such that they display CFU-A characteristics. However, further studies are needed to examine the origin of these colonies produced in the CFU-A assays in the absence of SCF.

10.2.2 The Effect of Altering SCF Levels on CFU-A Colony Formation

The reduction of SCF concentration from 12 ng/ml in ten fold steps has minimal effects on CFU-A colony formation, until the SCF concentration was reduced to 1.2

pg/ml, where CFU-A colony formation was reduced by 50% (figure 6.3). As mentioned in section 10.2.1, this may be explained by the decrease in SCFs ability to induce cell survival or even due to the reduction in synergistic interactions with the other CFU-A growth factors.

Interestingly, it was observed that a 10-fold increase in SCF above normal concentrations reduced the level of CFU-A colony formation (figure 6.3). Analysis of the constituent cells of the CFU-A colonies produced in assays containing high levels of SCF, indicated that high levels of SCF decreased the number of cells in the CFU-A colonies and also slightly increased the number of blast cells contained in these colonies (Table 4). Although SCF has been observed to display a limited ability to stimulate colony formation, a study by Metcalf *et al* observed that SCF could stimulate colony formation from bone marrow cells, but only at 1000 fold higher concentration than observed with other colony stimulating factors. Further analysis of these colonies indicated that they were small and contained anywhere in the region of 50-800 cells, and could be described as either blast, immature granulocytic or mature granulocytic colonies (Metcalf *et al* 1991a). This report provides evidence to suggest that high levels of SCF may support the formation of blast, immature granulocytic or mature granulocytic type of colonies within the CFU-A assays. Alternatively, the high levels of SCF may interact with the progenitor cells within the developing CFU-A colony and this may alter the constituent cells of the CFU-A colony. These hypothetical situations may explain the smaller colonies within the CFU-A assays that display a similar appearance to MIP-1α inhibited CFU-A colonies. Another report by Broxmeyer and colleagues suggested that, as the combination of GM-CSF and SCF increased the size of normal CFU-GM colonies compared to GM-CSF alone, SCF might serve to promote renewal and expansion of responsive progenitor cells within CFU-GM colonies (Broxmeyer *et al* 1991b). Therefore, it is possible that the high levels of SCF may produce a signal that can induce renewal of the CFU-A stem cells at the expense of differentiation, and this may account for the small increase in blast cells, and the decrease in CFU-A colony numbers observed in table 4. In an attempt to examine this possible self-renewal effect further, CFU-A colonies grown in the various SCF concentrations were picked and replated in a secondary CFU-A assay. The results from these assays were inconclusive, therefore, further analysis is needed to examine if high levels of SCF can reduce the CFU-A colony size by increasing self-renewal of the CFU-A stem cells at the expense of differentiation.

Alternatively, it was proposed that the reduction in the cellular content of the CFU-A colonies and the reduction in their colony numbers, as observed in table 4 and figures 6.3 and 6.5f, were due to the increase in expression of a growth inhibitor either directly or indirectly by the high levels of SCF. The mature cells within the bone marrow or the CFU-A colonies are not the only potential source of inhibitory molecules, as a recent study indicated that haemopoietic stem and progenitor cells also produce MIP-1α (Majka *et al* 1999). Therefore, it is possible that the bone marrow stroma cells, the mature blood cells or the progenitor cells can act as the source of inhibitory cytokines. However, experiments using a MIP-1α neutralising antibody, and bone marrow from MIP-1α null mice, indicated that the high levels of SCF did not produce the reduction in CFU-A colony number by increasing the production in MIP-1α (figure 6.7 and 6.9). Furthermore, expression studies also indicated that high levels of SCF could not induce MIP-1α expression in monocytic cell lines or bone marrow macrophages (figures 6.11, 6.12 and 6.13). Although these studies indicate that SCF does not reduce the CFU-A colony numbers, by increasing the expression of MIP-1α, it does not rule out the possibility that SCF may increase the expression of other growth inhibitory molecules such as TGF-β, TNF-α, IFN-α or MIP-1β. Therefore, further work is needed to examine the role of this high level of SCF in the reduction of CFU-A colony formation

Interestingly, SCF has also been observed to promote the production of IL-6 from mast cells, and a more recent study indicated that SCF could induce the expression of IL-6 in bone marrow stromal cells (Lu-Kuo *et al* 1996, Rougier *et al* 1998). IL-6 in combination with SCF has been observed to stimulate the production of blast cell colonies and granulocytic colonies (Tsuji *et al* 1991). Furthermore, data presented in table 8 also indicates that the addition of IL-6 to a CFU-A assay alters the constituent cells of the CFU-A colonies. Therefore, if SCF can induce the production of IL-6 in the bone marrow cells, it is feasible that the interaction of IL-6 and the CFU-A growth factors may alter the constituent cells of the CFU-A colonies, in such a way that they appear smaller. Alternatively as IL-6, M-CSF and GM-CSF, in combination with SCF, can support the formation of HPP-CFC colonies (Kreigler *et al* 1994). It is therefore possible that the combination of these growth factors may lead to the preferential production HPP-CFC colonies at the expense of CFU-A colonies.

As discussed above, there are several possible mechanisms that could have led to the reduction in the CFU-A colony formation in the assays containing high levels of SCF, and there are several experiments that could be performed to examine this effect further. The role of inhibitory molecules or growth factors in the reduction of the CFU-A colony formation could be analysed by adding specific neutralising antibodies to these growth factors or inhibitors to a CFU-A assay. A similar experiment using bone marrow from the null mice of growth factors suggested to be involved in the reduction of CFU-A colony numbers at high SCF concentrations (e.g. MIP-1α, IL-6), may also indicate whether these factors play a role in the decreased colony formation in assays containing high levels of SCF.

10.2.3 The Effect of Altering SCF on MIP-1α Activity

Reducing or increasing the concentration of SCF had no obvious effects on the overall ability of MIP-1α to inhibit the formation of CFU-A colonies (figure 6.5). Thus it appears that the SCF signalling pathway minimally interacts with the MIP-1α inhibitory signalling pathway.

Although the alteration of SCF concentration had no effect on MIP-1α inhibition of CFU-A colony formation, high concentrations of SCF in the presence of low concentrations of MIP-1α had a stimulatory effect on the numbers of CFU-A colonies (figure 6.5f). Despite this being the first report of MIP-1α stimulatory effects on CFU-A colony formation, stimulation of haemopoietic cells is not a new phenomenon for MIP-1α. Indeed, Keller *et al* previously reported that MIP-1α can stimulate progenitor colony growth, and this stimulatory activity is dependent upon the particular maturation stage of the stem/progenitor cell and the growth conditions (Keller *et al* 1994). Furthermore, Broxmeyer *et al* also reported that MIP-1α in combination with GM-CSF could enhance the colony growth of murine CFU-GM progenitors in normal mice (Broxmeyer *et al* 1989, 1990). Upon further examination of these effects, using bone marrow from CCR1 null mice, it was observed that MIP-1α did not enhance the growth of these CFU-GM progenitors, suggesting that the interaction between MIP-1α and CCR1 is essential for the growth promoting activities observed in the previous study (Broxmeyer *et al* 1999). As the interaction of MIP-1α and CCR-1 has been observed to play a stimulatory role in CFU-GM

colony formation, it is possible that this interaction may also stimulate CFU-A colony formation. Indeed, Jarmin *et al* reported that GM-CSF induces the production of CCR1 in bone marrow macrophages (Jarmin *et al* 1999). If high levels of SCF alone or in combination with GM-CSF can induce CCR1 expression on the CFU-A stem cells, it may be possible that the interaction of MIP-1α and CCR1 is involved in this growth promotion of the CFU-A colonies. Alternatively, CCR1 levels may be increased on the bone marrow progenitor cells, and upon the interaction with MIP-1α, this may induce progenitor colony growth to such an extent that these colonies display similar size characteristics as CFU-A colonies.

The models suggested above, do not explain why this increase in CFU-A colony numbers only occurs at low MIP-1α concentrations. However, there are a number of possible reasons why this may occur. It may be due to different signalling through the different MIP-1α receptors, such as CCR1 and the as yet unidentified MIP-1α inhibitory receptor. Indeed, low concentrations of chemotaxins have been observed to induce the characteristic feature of cell motility, whereas, high concentrations can induce the release of pro-inflammatory mediators, and this has been observed to be due to differential use of G-proteins (Haribabu *et al* 1999). This suggests that different concentrations of chemotaxins may activate different signalling pathways and this may explain the observation that high concentrations of MIP-1α inhibit CFU-A proliferation, whereas low concentrations are stimulatory in the presence of high SCF concentrations.

Interestingly, a report by Richardson *et al* indicated a further level of complexity in chemokine signalling. They observed that signalling through CXCR1 by IL-8 could cross desensitise the signalling potential through CCR1 (Richardson *et al* 2000). These reports described above, provide evidence that may be used to hypothesise on how MIP-1α can exert different growth activities. Furthermore, our group has observed that concentrations of MIP-1α lower than 50 ng/ml do not have the ability to inhibit CFU-A colony formation, suggesting the existence of an inhibitory concentration threshold that needs to be reached to activate the inhibitory signalling pathway. If upon activating this pathway, it has the effect of cross desensitising other MIP-1α signalling pathways, this may explain why high levels of MIP-1α only inhibit CFU-A formation and do not promote CFU-A colony growth. This

hypothetical situation may also explain the CFU-A growth promotion in CFU-A assays containing high levels of SCF or M-CSF, and low concentrations of MIP-1α. In these assays, the high levels of SCF or M-CSF alone or in combination with GM-CSF, may increase the levels of CCR1, a chemokine receptor previously observed to be involved in growth promotion (Broxmeyer *et al* 1999). The addition of low amounts of MIP-1α, which do not exceed the concentration threshold and thus do not activate the inhibitory signalling pathway, will bind to the increased levels of CCR1 and may lead to the observed increase in CFU-A colonies.

Further analysis of this growth stimulation is needed to examine whether this effect is regulated through the interaction of MIP-1α and CCR1. The role of CCR1 in the stimulation of these CFU-A colonies could be examined by the analysis of CFU-A colony formation from CCR1 null bone marrow. Alternatively, the use of a CCR1 antibody may also indicate that CCR1 is involved in the observed growth stimulation.

10.2.4 The Effect of Altering M-CSF Levels on CFU-A Colony Formation

Interestingly, the removal of M-CSF from the CFU-A assays produced a similar low level of CFU-A colony production, as observed in CFU-A assays containing no SCF (figure 6.4). The result of CFU-A colony formation in the absence of M-CSF concurs with a previous report by Pragnell *et al*, who observed that antisera to M-CSF was effective at inhibiting the production of CFU-A colonies by 92% (Pragnell *et al* 1988). These observations therefore suggest that M-CSF is an essential growth factor for CFU-A colony formation. A report by Horiguchi indicated that GM-CSF could induce the production of M-CSF mRNA and protein in monocytes (Horiguchi *et al* 1987). Therefore the residual level of CFU-A colony formation observed in figure 6.4 might be due to the induction of M-CSF in the bone marrow cells by GM-CSF. The reduction of M-CSF concentration in 10 fold steps from 6ng/ml to 6pg/ml was observed to produce a step wise reduction in CFU-A colony numbers, further indicating the importance of M-CSF in the formation of the CFU-A colonies (figure 6.4).

Similar to the high SCF concentrations, the increased levels of M-CSF were observed to reduce CFU-A colony formation, therefore, suggesting that this common

observation related to the fact that both of these growth factors signal through receptors that are members of the tyrosine kinase family. It was proposed that high levels of M-CSF produced its effects in a similar way to high levels of SCF. Therefore, the examination of the replating ability of CFU-A colonies, grown in high M-CSF, was performed. However, similar to assay containing high levels of SCF, the data produced was inconclusive and therefore, further replating experiments are needed to examine the potential role if any of high levels of M-CSF in self renewal of CFU-A stem cells. Alternatively, it was proposed that this reduction in colony formation, observed in high M-CSF concentrations, was due to the production of an inhibitory cytokine. Indeed, M-CSF is capable of inducing the expression of MIP-1α in bone marrow macrophages Jarmin, personal communication. Furthermore, M-CSF has also been observed to induce the expression of several growth factors, cytokines and inhibitory molecules such as IL-6, G-CSF, GM-CSF, M-CSF, IFN and IL-8 from human monocytes (Motoyoshi *et al* 1989, Kamdar *et al* 1997, Warren *et al* 1986, Hashimoto *et al* 1996). M-CSF has also been reported to induce the expression of TNF-α and increase mRNA levels of TGF-β1 (Sakurai *et al* 1994, Wu *et al* 1997). Therefore, it is possible that high levels of M-CSF may produce the reduction in CFU-A colonies by the direct induction of expression of growth inhibitors. Alternatively, M-CSF may act on the various cell types within the bone marrow to induce the expression of other cytokines, who may be able to induce the expression of inhibitory molecules. Studies using MIP-1α neutralising antibodies and bone marrow from MIP-1α null mice indicated, that this reduction in CFU-A colony numbers in high M-CSF conditions was not due to the increased production of MIP-1α (figure 6.8 and 6.10). Although these studies indicate that high levels of M-CSF do not increase the expression of MIP-1α, it does not rule out the possibility of high levels of M-CSF inducing the production of other inhibitory molecules.

10.2.5 The Effect of Altering M-CSF Levels on MIP-1α Activity

Similar to the alteration in SCF concentration, reducing or increasing the concentration of M-CSF had no obvious effects on the overall ability of MIP-1α to inhibit the formation of CFU-A colonies (figure 6.6). Pragnell previously reported data that CFU-A colonies are predominantly made up of macrophages (Pragnell *et al* 1994) and this was confirmed by data reported in table 5. Similar to high levels of

SCF, high levels of M-CSF were observed to reduce the CFU-A colony formation, and in conjunction with low levels of MIP-1α, high levels of M-CSF have been shown to stimulate CFU-A colony formation. This data therefore suggested that these similar effects observed with SCF and M-CSF may be produced via similar signalling pathways.

The further analysis of the role M-CSF plays in the CFU-A assays would be similar to the ones suggested in sections 10.2.2 and 10.2.3. The ability of M-CSF to reduce CFU-A colonies, by the induction of expression of other growth regulators, could be examined by the incorporation of neutralising antibodies to these factors within the CFU-A assays. Alternatively, using bone marrow from these growth regulator null mice similar experiments could be performed. Furthermore, in section 10.2.4 it was suggested that GM-CSF could induce the expression of M-CSF, this effect may explain the CFU-A colony formation in the absence of M-CSF. This effect could be analysed further by the examination of CFU-A colony formation from the bone marrow of *op/op* mice in the absence of M-CSF.

10.2.6 The Effect of Alterating GM-CSF Levels on CFU-A Colony Formation and MIP-1 α Activity

The omission of GM-CSF from the CFU-A assays results in the inability of CFU-A stem cells to produce colonies indicating that GM-CSF is essential for the formation of CFU-A colonies (figure 7.1). Furthermore, the essential role of GM-CSF was confirmed upon the observation that 1000 fold lower than normal levels of GM-CSF could not promote CFU-A colony formation, and CFU-A colonies were produced only after a further 10 fold increase in GM-CSF concentration. Similar to SCF and M-CSF, the alteration of GM-CSF concentration displayed a minimal affect on the inhibitory activity of MIP-1α therefore suggesting that GM-CSF, like SCF and M-CSF, signalling pathways are limited in their interaction with the MIP-1α inhibitory signalling pathways.

Upon analysis of CFU-A assays containing 10 and 100 fold normal GM-CSF concentrations in the presence of 50 ng/ml of MIP-1α, an interesting alternative colony shape was consistently observed. These colonies are similar in size to normal

CFU-A colonies, however, they displayed a more defined edge compared to the normal CFU-A colonies (figure 7.3 A and B). On further analysis of the assay plates containing the alternative colonies, it was observed that these alternative colonies appeared half-spherical in shape and, unlike normal colonies, were embedded in the two layers of the CFU-A assay. Furthermore, these alternative colonies have a defined central region, and what appears to be a leading edge of cells, that may be produced by cells moving away from the central area (figure 7.4 C and D). Jarmin *et al* reported that GM-CSF and IL-3 could enhance the expression of CCR1 on the surface of bone marrow macrophages (Jarmin *et al* 1999). Interestingly, a possible role for CCR1 in growth promotion was observed by Broxmeyer *et al* who reported that CCR1 is the receptor that is involved in the stimulation of CFU-GM colony formation by MIP-1α (Broxmeyer *et al* 1999). Therefore, it may be possible that the interaction of MIP-1α with the increased levels of CCR1, induced by high levels of GM-CSF, may be involved in this alternative CFU-A colony shape.

In an attempt to examine if CCR1 was involved in the formation of these alternative colonies, HepMut (a variant of MIP-1α) was added to CFU-A assays containing high levels of GM-CSF. Graham *et al* previously reported that HepMut was as potent as MIP-1α at inhibiting CFU-A colony formation, however, unlike MIP-1α, it could not bind to CCR1 (Graham *et al* 1996). Although HepMut was observed to inhibit CFU-A colony formation (figure 7.5), it did not produce the alternatively shaped colonies, suggesting that CCR1 is involved in the formation of the alternative colonies (data not shown). Further analysis of other CCR1 ligands revealed that RANTES and MCP-3 were unable to induce the formation of these alternative colonies. However high levels of HCC-1 were observed to induce the formation of these alternative colonies as well as inhibit the formation of CFU-A colonies (figure 7.8). The inability of the CCR1 ligands RANTES and MCP-3 to induce the formation of these alternative colonies suggests that MIP-1α and HCC-1 somehow specifically induce the formation of these alternative colonies. This may be through an unknown CC chemokine receptor that is restricted to interact with MIP-1α and HCC-1, and is up regulated by GM-CSF. However, further evidence to suggest that CCR1 is involved in the formation of these alternative colonies is described in chapter 9. The expansion of bone marrow with SCF and IL-11 for 6 days was observed to increase the expression of CCR1 (figure 9.6). Upon the incubation of these expanded cells in a CFU-A assay

containing MIP-1α, it was observed that the alternative colonies were produced at all concentrations except 100 ng/ml of MIP-1α (data not shown). This provided further evidence to suggest that the interaction of MIP-1α with the possible increased levels of CCR1, due to GM-CSF treatment, may be involved in the formation of these alternative colonies.

In a further attempt to understand how these alternative colonies are formed, an analysis of the constituent cells of these alternative CFU-A colonies was performed and the results in table 6, indicate that similar to normal CFU-A colonies, these alternative colonies predominantly consist of macrophages. However, interestingly the alternative colonies have a 4 % increase in the number of granulocytes compared to the normal CFU-A colonies when incubated in 10 x the normal levels of GM-CSF. Previous characterisation of CFU-GM colonies indicated that they have a tight central area consisting of neutrophils surrounded by a halo of macrophages. If as suggested by analysis of CFU-GM colonies the increase in granulocytes occurs at the central region of these alternative CFU-A colonies, this may explain the appearance of a defined central area (figure 7.4C and D). These centrally located granulocytes may also be the key to the other characteristic feature of these colonies i.e. the leading edge of cells that appear to be moving away from the centre of the colony. Indeed, a recent study by Cheng *et al* demonstrated that GM-CSF could induce the expression of CCR-1 on neutrophils (Cheng *et al* 2001). Therefore it may be possible that the stimulation of these granulocytes by MIP-1α, through CCR1, enables these cells to produce some factor that repels the macrophages from the central region of these colonies and this in turn may lead to the formation of the outer edge of these alternative colonies. Chemokines are generally accepted as molecules that can induce movement of various types of leukocytes, whether it is random movement (which is referred to as chemokinesis) or controlled movement through a concentration gradient (which is known as chemotaxis). Until recently, chemokines have never been shown to induce cellular repulsion however, Poznansky *et al* observed that high concentrations of SDF-1 can induce the repulsion of T cells *in-vitro* and *in-vivo* (Poznansky *et al* 2000). They referred to this cell movement away from a high concentration of chemotactic agent as "chemofugetaxis".

From the various studies described above, a possible model for the generation of the alternative colonies is suggested below. Firstly, the high levels of GM-CSF are proposed to induce the expression of CCR1 on the centrally located granulocytic cells of the CFU-A colony. Upon the interaction of MIP-1α with these increased levels of CCR1, it is proposed that this induces these cells to produce a repelling factor, e.g. SDF-1. This may therefore produce a high local concentration, which upon interaction with CXCR4 bearing cells (predominantly macrophages in CFU-A colonies), induces these cells to move away from this high local concentration. SDF-1 has been observed to chemoattract neutrophils, monocytes and T cells (Oberlin *et al* 1996), and with the recent observation that SDF-1 can repel T cells it may be feasible that this repellent property may be seen with other SDF-1 receptor expressing cells. Therefore, it is possible that SDF-1 or some other repelling factor is involved in the movement of cells and thus may explain the appearance of the alternative colonies. Indeed, the highly stained central region may consist of cells that are CXCR4 negative and thus are not repelled, whereas the highly stained outer edge may consist of CXCR4 positive cells that have moved away from the high local concentration in the central area of the colony.

To test the above hypothesis, it would be prudent to design experiments to analyse the various roles that the suggested factors play in the formation of these alternative colonies. There are a number of experiments that can examine the individual roles of these factors. Indeed neutralising antibodies to SDF-1 could examine its possible role in the repulsion of the cells in these colonies. Alternatively, the basis for these colonies could be examined by investigating the CFU-A colony formation from the bone marrow of CCR1 null mice. These experiments would hopefully clarify the roles that CCR1 and SDF-1 play in the formation of these alternative colonies. Furthermore the role the constituent cells play in the formation of the alternatively shaped colonies may be examined by staining them with esterases to examine the localisation of the neutrophils and macrophages within these colonies.

10.3 The Effects of the Addition of gp130 Cytokines to the CFU-A Assay

Glycoprotein 130 cytokines are not required for the formation of CFU-A colonies however upon the addition of IL-11 or IL-6 to a CFU-A assay, it was observed that both of these cytokines could reduce the formation of CFU-A colonies. Although IL-

6 and IL-11 have never been reported to inhibit the formation of CFU-A colonies, or indeed the formation of any stem or progenitor colonies, it is possible that they can either inhibit the formation of CFU-A colonies directly or via the expression of an inhibitory molecule. Further analysis of the colonies grown in IL-6 and IL-11 revealed that high levels of IL-11 and IL-6 both increased the number of granulocytes within the colonies, and also that high levels of IL-6 increased the number of blast cells within the CFU-A colonies (Table 7 and 8). This therefore suggested that the combination of the CFU-A growth factors and IL-6 or IL-11 altered the differentiation of CFU-A stem cells, thus producing smaller colonies. Interleukin-6 in combination with SCF has been observed to induce the formation of blast cell colonies, HPP-CFC colonies and CFU-GM colonies (Kreigler *et al* 1994, Sui *et al* 1995). Similar to IL-6, IL-11 can support granulocyte/macrophage colonies, and furthermore, the administration of IL-11 to mice has been observed to increase the numbers and cycling rate of CFU-GM and CFU-GEMM (Musashi *et al* 1991, Hangoc *et al* 1993).

As indicated in table 8, the addition of IL-6 to CFU-A assays increases the number of blast cells within the CFU-A colonies. It was hypothesised that these blast cells may be CFU-A stem cells, however, on the analysis of the replating potential of these blast cells it was observed that they do not produce secondary CFU-A colonies (data not shown). This indicated that although the high levels of IL-6 induced an increase in blast cells within the CFU-A colonies, they are not CFU-A stem cells and thus, further analysis is needed to identify these blast cells.

The addition of a further gp130 signalling cytokine leukaemia inhibitory factor (LIF) to the CFU-A assay had no effect on colony numbers or MIP-1α inhibition. This inability of LIF to affect CFU-A colony formation may be explained by the observation that short term repopulating cells, which contain CFU-A stem cells, do not have the ability to bind radiolabelled LIF, suggesting that these cells do not have LIF receptors (McKinstry *et al* 1997). A contradictory study by Imamura *et al* the observed that SCF and LIF expanded the number of transiently engrafting stem cells from bone marrow, suggesting that these cells did in fact express LIF receptors (Imamura *et al* 1996). However, it is possible that this increase in transiently engrafting stem cell amplification is due to the expansion of pre-CFU-A stem cells and not due to a direct amplification of the CFU-A stem cells themselves.

10.3.1 The Effect of IL-11 and IL-6 on MIP-1α Inhibition of CFU-A Colony Formation

Upon the inclusion of various concentrations of MIP-1α to CFU-A assays containing IL-6, it was observed that MIP-1α at 10 ng/ml, a concentration not normally associated with inhibition of CFU-A colony formation, could inhibit CFU-A colony formation in assays containing IL-6. It is possible that this is a direct inhibitory effect of MIP-1α in these assays. However, a study by Fahey *et al* observed that 100 ng/ml of MIP-1α induced the expression of 1 ng/ml of IL-6 from cultured macrophages after 6 hours, therefore, indicating that it is entirely feasible that the further reduction in the colony formation is due to an additive effect of the exogenously added IL-6 and the endogenously induced IL-6 (Fahey *et al* 1992). Interestingly as mentioned earlier SCF has also been observed to induce IL-6 expression in bone marrow stroma therefore it is possible that, in combination with MIP-1α, SCF also increases the production of IL-6 from the bone marrow cells (Rougier *et al* 1998). On analysis of the replating ability of CFU-A colonies grown in high

Further analysis is needed to examine whether the reduction in CFU-A colonies observed in assays containing IL-6 and MIP-1α is through the induction of MIP-1α by IL-6 or due to the induction of IL-6 by MIP-1α. The analysis of the CFU-A colony forming potential of bone marrow from MIP-1α and IL-6 null mice plated in assays containing IL-6 and/or MIP-1α may clarify these observations. Alternatively, MIP-1α neutralising antibodies would also clarify this matter.

10.4 Ex-vivo Expansion

As described in the sections above, CFU-A growth factors had no effect on MIP-1α inhibition of CFU-A colonies. Therefore, in a further attempt to examine the effect of growth factors on the inhibition of proliferation by MIP-1α an assay, unlike the CFU-A assay, which allows the differentiation of CFU-A stem cells into CFU-A colonies, was needed.

The *ex-vivo* expansion of stem cells is an assay system where cocktails of growth factors induce the expansion of various stem cell populations prior to transplantation.

It was initially thought that this expansion was due to self-renewal of the various stem cell populations, however, expansion protocols invariably lead to an increase in mature cell numbers and a decrease in LTR ability, indicating that the increase in cell number is likely not to be via self-renewal of stem cells.

Numerous *ex-vivo* expansion studies have been performed using various combinations of growth factors. Indeed Bodine *et al* reported that various combinations of growth factors can induce the expansion of CFU-S stem cells *in-vitro,* and upon the addition of MIP-1α, the increase in CFU-S numbers was reduced (Bodine *et al* 1991). Similarly, Holyoake *et al* also observed that the combination of growth factors, such as SCF and IL-11, could amplify the transiently engrafting stem cells (CFU-A) by 50 fold. However, unlike the Bodine study, the addition of MIP-1α to the expansion protocol did not decrease or further increase the CFU-A numbers (Holyoake *et al* 1996).

In this thesis, SCF and IL-11 were observed to amplify the CFU-A numbers by 30 fold and similar to the report by Holyoake *et al*, the addition of MIP-1α to the expansion protocol did not reduce or increase this level of expansion (figure 9.1). The effect of MIP-1α on the formation of CFU-A colonies from normal and expanded bone marrow was next examined. After 6 days of expansion in SCF and IL-11, the potential of MIP-1α to inhibit the colony formation was assessed. Unlike the 80 % inhibition observed upon the addition of MIP-1α to normal bone marrow, MIP-1α could only reduce the CFU-A colony formation by 10-20% using expanded bone marrow. This indicated that the expansion of bone marrow with SCF and IL-11 was interfering with the inhibitory properties of MIP-1α. To investigate if this effect was specific to MIP-1α, the ability of TGF-β, a previously reported inhibitor of CFU-A colony formation (Maltman *et al* 1996), to inhibit CFU-A colony formation from expanded bone marrow was analysed. After a 6 day expansion of bone marrow with SCF and IL-11, TGF-β was observed to inhibit the formation of CFU-A colonies from the expanded bone marrow (figure 9.5) to a similar degree, as observed with normal bone marrow. This indicated that the expansion of the CFU-A stem cells by SCF and IL-11 somehow interfered with the inhibitory activity of MIP-1α and this was proposed to be due to the down regulation of MIP-1α receptors. Alternatively, SCF or IL-11, alone or together, may indirectly regulate the expression of chemokine

receptors by inducing the expression of other cytokines that can reduce or down regulate the expression of the MIP-1α receptors. Indeed TNF-α, IL-1, IFN, IL-10, IL-12 and IL-16 are a few of the cytokines that have been reported to regulate the expression of MIP-1α receptors. As SCF can induce the expression of IL-1 and TNF-α, it is possible that the indirect action of SCF may result in the reduction in receptor levels.

Ex-vivo expansion protocols have been observed to increase the numbers of mature cells, therefore, prior to the examination of the expression of mRNA for the known MIP-1α receptors CCR1, CCR3 and D6, a lineage depletion of both the expanded and non expanded cellular populations was performed. The inclusion of a lineage depletion step will produce a more comparable starting cellular population. Expression of messenger RNA for the known MIP-1α receptors was examined and it was observed that CCR1 was up regulated whilst CCR3 and D6 were down regulated in the lineage depleted expanded bone marrow compared to the lineage depleted non expanded bone marrow. These results suggested that maybe CCR3 and D6 are involved in the inhibition of CFU-A stem cells (figure 9.7). However, unpublished data within our group using bone marrow from D6 null mice indicated that MIP-1α can still inhibit the formation of CFU-A colonies, therefore suggesting that D6 is not involved in MIP-1α inhibition of CFU-A colony formation. Data from figure 9.8 indicates that a high excess of eotaxin cannot stop MIP-1α inhibiting CFU-A colony formation thus demonstrating that CCR3 is unlikely the receptor involved in the inhibition of CFU-A stem cells (figure 9.8). Thus although CCR3 and D6 are all down regulated none of them account for the inhibitory activity of MIP-1α thus further studies are needed to identify for this MIP-1α inhibitory receptor. However to rule out CCR3 as the inhibitory receptor the analysis of bone marrow from CCR3 null mice in the CFU-A assay is needed.

10.5 Summary

MIP-1α is a member of a group of pro-inflammatory molecules known as chemokines, the various members of which display a multitude of activities including development, angiogenesis and regulation of specific leukocyte trafficking (Streiter *et al* 1995, Sozzani *et al* 1997). Furthermore MIP-1α has been observed to be involved

in various inflammatory diseases such as EAE and asthma. However the main activity that this thesis is concerned with is MIP-1α's ability to inhibit the proliferation of the transient engrafting stem cells, namely the CFU-S and CFU-A stem cells. This is of particular interest as it has been estimated that in steady state conditions, it is likely that the majority of haemopoietic function is regulated at the level of the transiently engrafting stem cells with little input from the primitive long term repopulating cells. Therefore, it is likely that the transient engrafting stem cells, the target of MIP-1α, may be more in need of regular growth control. Although this may be the case there is a lack of reports investigating how MIP-1α exerts its inhibitory activity, therefore this thesis was undertaken to investigate if altering the CFU-A growth conditions would interfere with MIP-1α inhibition of CFU-A colony formation, and thus allow us to infer modes of action of MIP-1α.

In-vitro stem and progenitor assays by definition identify a particular stem or progenitor cell by the formation of mature cell colonies, however, there are aspects of these assays that are not fully understood. Within these assays it is not known what effect the varied cellular populations within the bone marrow play in the colony formation or indeed, the exact role of the various growth factors have in the formation of these colonies . All we know for certain is that the various stem/progenitor cells will differentiate into their respective colonies in assays conatining specific growth conditions.

The data presented in this thesis confirmed that CFU-A stem cells require SCF, M-CSF and GM-CSF to produce their characteristic colonies and that MIP-1α inhibits the formation of these colonies. Furthermore the alteration of SCF, M-CSF and GM-CSF concentration and the addition of IL-11 or LIF to the assay had minimal effects on the inhibition of the CFU-A colony formation by MIP-1α. Although the addition of IL-6 to the CFU-A assay seemed to increase the inhibitory activity of MIP-1α , it is possible that this effect may be due to the altered differentiation of the CFU-A stem cells as suggested by the increase in neutrophils and blast cells, (table 8).

Although the alteration of growth factor concentration did not interfere with thre inhibitory activity of MIP-1α, it did however interact with other MIP-1α signalling pathways. Indeed high lebvels of SCF and M-CSF, in the presence of low levels of

MIP-1α were observed to produce an increase in CFU-A colony formation. Furthermore the combination of high levels of GM-CSF and 50ng/ml of MIP-1α lead to the production of an alternatively shaped CFU-A colonies. These effects were hypothesised to be due to the interaction of MIP-1α and one of its receptors CCR-1. Indeed, Broxmeyer *et al* observed that CCR-1 is involved in the growth of CFU-GM progenitors (Broxmeyer *et al* 1999), therefore it may also be involved in the increase in the CFU-A colony formatiom. Futhermore GM-CSF has been observed to increase the expression of CCR-1 on macrophages (Jarmin *et al* 1999), therefore the analysis of CCR-1 null mice bone marrow in these assays is needed to examine the role, if any, that CCR-1 plays in these observations.

There are other interesting observations that merit further investigartion, indeed, it was observed that upon the removal of either SCF, M-CSF or GM-CSF from the CFU-A assay that various sized colonies were formed. The clonal origin of these colonies is uncertain, however, in assays containing either no SCF or no M-CSF colonies that displayed similar size characteristics to CFU-A colonies were observed. It is entiorely feasible that these colonies are CFU-A colonies as Pragnell previously observed that M-CSF and GM-CSF are sufficient fir CFU-A colony formation (Pragnell et al 1994). Furthernore GM-CSF has been observed to induce M-CSF expression in bone marrow cells, it is feasible that this makes up for the omission of M-CSf and results in CFU-A colony formation (Horiguchi et al 1987).

One other area worthy of further investigation is the effect the expansion of bone marrow by SCF and IL-11 treatment has on the CFU-A stem cells. Indeed, ex-vivo expansion of bone marrow not only altered the chemokine receptor levels, it also aided the formation of the alternative colony shape and reduced the effectiveness of MIP-1α at inhibiting CFU-A colony formation .

These results indicate that in the context of the CFU-A assay the MIP-1α inhibitory signalling pathway is robust and may only minimally interact with the CFU-A growth factors, IL-11 or LIF signalling pathways. However data also suggests that a different MIP-1α signalling pathway may interact with the tyrosine kinase signalling pathways, leading to an increase in CFU-A colony growth. Although growth factor signalling pathways in the CFU-A assays cannot interfere with MIP-1α inhibitory signalling pathway that is not the case in ex-vivo expansion assays. This suggest that the

interaction of the growth factors signalling pathways and MIP-1α signalling pathways are dependent on the assay type and possibly the particular function the growth factors have in the particular assays. In altering the growth conditions of the CFU-A assay we have attempted to interfere with MIP-1α inhibitory induced signalling, it is noted that these types of experiments may not be the best model to use However it is possible that they may aid to direct further analysis of signalling interactions via other methods such as proteomics.

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