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Homing and engraftment of $CD34^+$ cells in the NOD/SCID model

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This thesis is dedicated to
my father and mother

ABSTRACT

The reduced engraftment potential of haemopoietic stem/progenitor cells after exposure to cytokines may be related to the impaired homing ability of actively cycling cells. I tested this hypothesis by quantifying the short-term homing of human adult $CD34^+$ cells in NOD/SCID animals. I have demonstrated in adult $CD34^+$ cells that cytokine exposure ex-vivo leads to a loss of engraftment ability *in vivo* which occurs rapidly and which is associated with a striking alteration in the tissue distribution of homed cells. Loss of homing to the BM and to a lesser extent, the spleen, coincides with increased accumulation of cells in the lungs. The loss of BM homing and engraftment is not related to cell cycle phase, and was not restored by blocking Fas ligation *in vivo*. The co-transplantation of adult MPB $CD34^+$ cells with non-haemopoietic stem cells (e.g. MSCs and MAPCs) and heterologous T cells (e.g. $CD4^+$ and $CD8^+$) was also unable to improve the homing to the BM in the NOD/SCID mice. The work from this thesis suggest that these changes in homing, and as a consequence engraftment, induced by cytokine exposure are due to increased migratory capacity of infused activated cells, leading to loss of selectivity of the homing process.

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List of Abbreviations used:

BFU-E	"Burst"-Forming Unit for Erythroid cells
BM	Bone Marrow
BMMNC	Bone Marrow MonoNuclearCell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFC	Colony-Forming Cell
CFU-GM (GM-CFC)	Colony-Forming Unit for Granulo-Monocytic cells
CO_2	Carbon Dioxide
ECGS	Endothelial Cell Growth Supplement
EDTA	Ethylenediaminetetraacetic acid-dipotassium salt
EGF	Epidermal growth factor
EPO	Erythropoietin
FACS	Fluorescence activated cell sorter
FCS	Foetal Calf Serum
Flt3L	Fms-like tyrosine kinase receptor ligand
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CFC (CFU-GM)	Granulocyte-Monocyte Forming Cell (as for CFU-GM)
GM-CSF	Granulocyte-Monocyte-Colony Stimulating Factor
HBSS	Hanks Buffered Saline Solution
HSPC	Haemopoietic Stem Progenitor Cell
H_2O_2	Hydrogen peroxide
HPC	Haemopoietic Progenitor Cell
Hst	Hoechst 33342
HUVEC	Human Umbilical Vein Endothelial Cells
IL-3	InterLeukin-3 (Other cytokines include IL-6, IL-11)
ITS	Insulin-transferrin-sodium selenite
IMDM	Iscove's Modified Dulbecco's Medium
LFA-1, LFA-2	Leukocyte-Activating Factor-1,2
LTCIC	Long-Term Colony Initiating Cell
LTRC	Long Term Repopulating Cell
MFI	Mean Fluorescence Intensity
MGG	May-Grunwald and Giemsa stain
MIP-1α	Macrophage Inhibitory Protein-alpha
MPB	Mobilised Peripheral Blood
NOD/SCID	Non-obese diabetic/severe combined Immunodeficient
NOD/SCID β2m	Non-obese diabetic/severe combined Immunodeficient β2 microglobulin
PBPC	Peripheral Blood Progenitor Cells (also PBSC)
PBS	Phosphate-Buffered Saline
PBSC	Peripheral Blood Stem Cells (also PBPC)
PDGF	Platelet Derived Growth Factor
PECAM-1	Platelet Endothelial Adhesion Molecule-1
PI	Propidium iodide
RPMI	Roswell Park Memorial Institute Media
SCF	Stem Cell Factor (c-kit ligand)
SDF-1	Stromal Derived Factor-1
STRC	Short Term Repopulating Cell
TBI	Total Body Irradiation
TBS	Tris Buffered Saline
TPO	Thrombopoietin (c-mpl ligand)
UCB	Umbilical Cord Blood
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very-Late Antigen-4
VLA-5	Very-Late Antigen-5

Chapter 1: Introduction

1.1 Haemopoiesis: an overview

Haemopoiesis is the process of blood cell formation. Haemopoiesis is a highly orchestrated process and each day the human body produces billions of new white blood cells, red blood cells, and platelets to replace blood cells lost to normal cell turnover processes as well as illness and trauma. The total cellular content is estimated at around $2x10^{12}$ cells with a daily turnover of $2x10^{11}$ red blood cells, $1x10^{10}$ white cells and $4x10^{11}$ platelets (Tavassoli, 1980). The vast majority of haemopoietic cells are morphologically recognisable and mature end-cells but within the marrow there are much smaller sub-populations of more primitive multipotent and lineage restricted cells (around $1\text{-}10x10^{6}$, (Moore, 1996)) which are morphologically similar in appearance to that of small or intermediate lymphocytes.

Haemopoietic stem cells (HSCs) generate the multiple haemopoietic lineages through a successive series of intermediate progenitors. These include common lymphoid progenitors (CLPs), which can generate only B, T and NK cells, and common myeloid progenitors (CMPs), which can generate only red cells, platelets, granulocytes, and monocytes (Akashi *et al*, 2000;Kondo *et al*, 1997). Downstream of the CLPs and CMPs are more mature progenitors that are further restricted in number and type of lineages that they can generate (Akashi *et al*, 2000). Ultimately terminally differentiated cells are produced that cannot divide and undergo apoptosis after a period of time ranging from hours (e.g. neutrophils 1 hr) to days (e.g. erythrocytes 120 days) in humans. A schematic representation of haemopoietic lineage development in the bone marrow is shown in Figure 1.1.

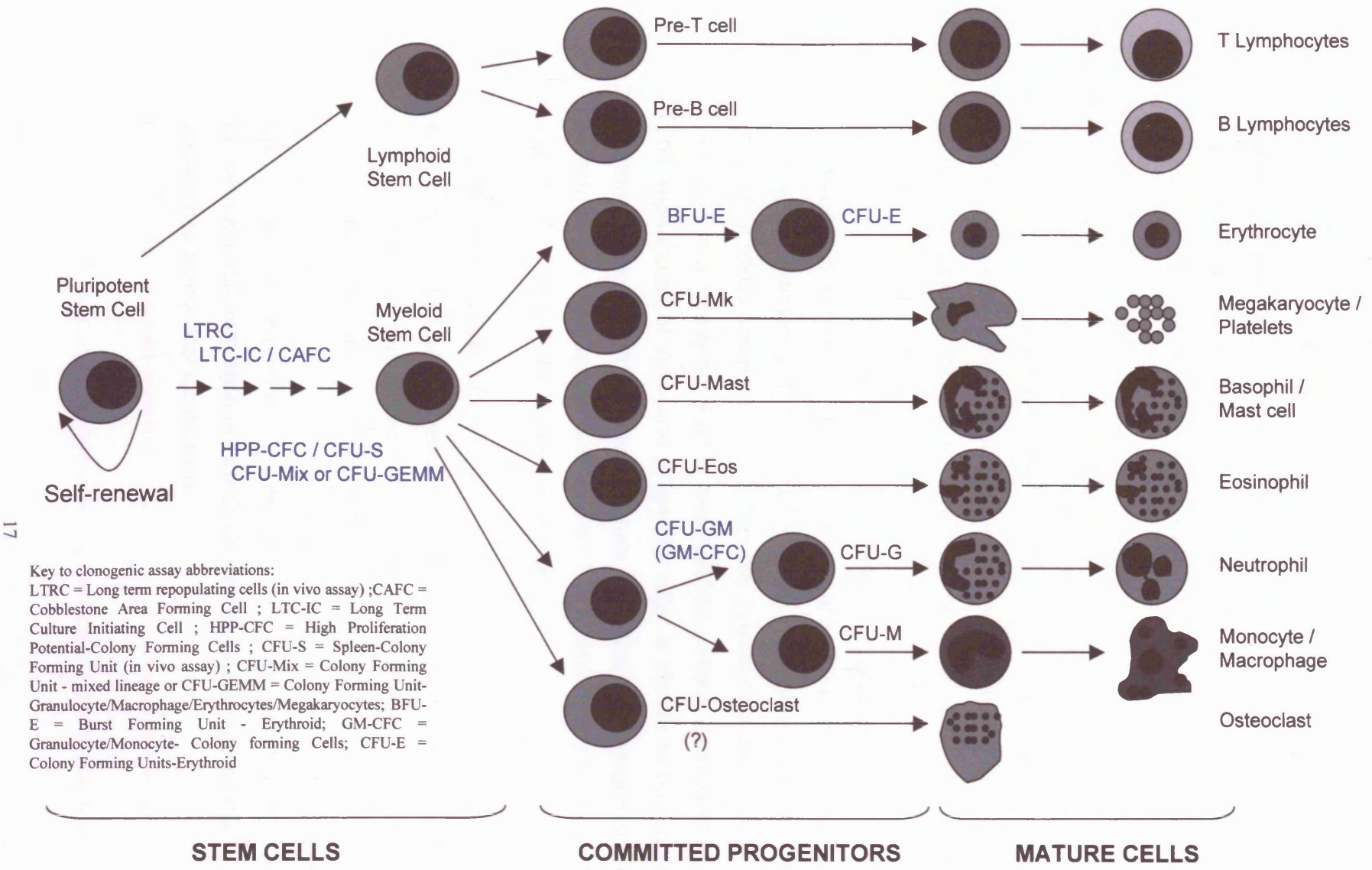


Figure 1.1 - Schematic representation of haemopoietic cell development and haemopoietic assays (highlighted in blue)

1.1.1 Sites

The sites of haemopoiesis in foetal life are the yolk sac (0-2 months), and later the liver and spleen (2-7 months). The bone marrow is the most important site from 6-7 months of foetal life (Tavassoli & Minguell, 1991;Tavassoli, 1991) and, after birth normal haemopoiesis is restricted to the bone marrow. Infants have haemopoietic marrow in all bones but in adults it is confined to axial skeleton and proximal ends of the femurs and humeri.

1.1.2 Haemopoietic stem and progenitor cells (HSPCs)

The best characterised stem cells are those responsible for haemopoiesis. HSCs have certain hallmark properties and they include the following: HSCs have the capacity for self-renewal, proliferation and differentiation to increasingly specialised progenitor cells which, after many cell divisions within the marrow, form mature cells (red cells, granulocytes, monocytes, platelets, and lymphocytes) of the peripheral blood (Figure 1.1). HSCs are rare, with a frequency of 1 in 10,000 to 100, 000 total blood cells and the majority of HSCs and many progenitors are quiescent in the G_0 phase of the cell cycle in a steady-state adult haemopoietic system (Uchida *et al*, 1997). The appearance of stem and progenitor cells is similar to that of small or intermediate sized lymphocytes, (Figure 3.1), however, progenitors can be detected by *in vitro* assays (see section 1.3) in which they form colonies for e.g. GM-CFC and BFU-E in semi-solid media.

1.1.3 Bone marrow stroma

After birth normal haemopoiesis is restricted to the bone marrow. The bone marrow is composed of stromal cells (e.g. fibroblasts, endothelial cells, macrophages, fat cells) and extracellular matrix (e.g. fibronectin, laminin, collagen, proteoglycans) and forms a suitable environment for stem cell growth and development, providing supportive signals for survival, proliferation, differentiation of HSPC, as well as anchorage points to ensure appropriate anatomical localisation.

1.1.4 Haemopoietic growth factors

Haemopoietic growth factors and cytokines are glycoproteins that regulate the proliferation and differentiation of haemopoietic progenitor cells and function of mature blood cells (Table 1.1). The major cell sources for these growth factors are T cells, monocytes and macrophages and the stromal cells (fibroblasts and endothelial cells) found in the BM except for EPO, 90% of which is synthesised in the kidney. Key cytokines for the expansion of progenitor cells are SCF and Flt3L (Williams *et al*, 1992;Rosnet *et al*, 1993;Verfaillie *et al*, 2000) as outlined below (see section 1.2) and discussed in detail in Chapter 5.

Table 1.1 - Haemopoietic growth factors (adapted from (Hoffbrand & Pettit, 1993))

Act on cells	Growth factors
Stromal cells	IL1, TNF
Pluripotent cells	SCF (stem cell factor, c-kit ligand, Steel factor), Flt3L
Early multipotential cells	IL-3, IL-6, GM-CSF
Late cells committed to one (or two) lineages	G-CSF, M-CSF, IL-5, Epo, TPO

1.2 Cytokines and *ex vivo* expansion

Many investigators are now attempting *ex vivo* manipulation of HSCs for potential therapeutic purposes for example expansion of small umbilical cord blood grafts to expand HSCs responsible for long term haemopoietic reconstitution for HSC transplantation. Gene transduction also requires cytokine stimulation. Below is a list of the major cytokines used for expansion of human HSCs (reviewed in (Heike & Nakahata, 2002)). However, the optimal choice of cytokine combinations for the *ex vivo* expansion of human HSPCs has not yet been determined and is discussed in detail in Chapter 5.

1.2.1 Stem cell factor (SCF)

SCF also known as c-Kit ligand is the ligand for the tyrosine kinase receptor c-Kit, which is expressed on both primitive and mature haemopoietic progenitor cells (Galli *et al*, 1994;McNiece & Briddell, 1995). SCF plays a central role in the regulation of early haemopoiesis (Andrews *et al*, 1994). SCF has been studied extensively in *ex vivo* expansion experiments and was frequently represented in the most effective combinations. The addition of SCF to combination of IL-3 and GM-CSF resulted in a 12 fold increase in the numbers of HPP-CFC derived colonies. SCF resulted in the increase of BFU-E, CFU-GM and BFU-MK colonies (Brandt *et al*, 1992;Hoffman *et al*, 1993).

1.2.2 Fms-like tyrosine kinase receptor ligand (Flt3L)

Flt3L is the ligand for the tyrosine kinase receptor Flt3 and as with KL, Flt3L affects proliferation and differentiation of primitive progenitors *in vitro*. Flt3L is efficacious in prompting stem cell cycling with retention of self renewal, particularly when combined with TPO, and SCF (Petzer *et al*, 1996). In a primate study the addition of Flt3L and stromal support to a 4 day cytokine priming with IL-3, IL-6 and KL alone, increased the degree of subsequent long term engraftment of retrovirally transduced cells from 0.01% to a clinically relevant 10-20% (Dunbar *et al*, 2001).

1.2.3 Interleukin-3 (IL-3)

IL-3 has a broad target specificity and is used in a number of cytokine combinations used for *ex vivo* expansion. The contribution of IL-3 for *ex vivo* expansion of HSCs remains controversial and is discussed in detail in Chapter 5. The study by Peters *et al.,* (1996) showed that stimulation with IL-3 may result in decreased ability of stem cell repopulation to self renew as assessed by long term repopulating capacity (Peters *et al*, 1996;Yonemura *et al*, 1996).

1.2.4 Interleukin-6 (IL-6)

IL-6 is member of the IL-6 cytokine family and these cytokines mediate their biologic action through the common subunit gp130. IL-6 alone does not have distinct biological activity on HSC expansion, however, it has been shown to

act as a potent co-factor in the expansion of human $CD34^+$ progenitor cells *in vitro* (Moore *et al*, 1997;Bernad *et al*, 1994;Shah *et al*, 1996). Therefore IL-6 acts in synergy with the SCF, Flt3L to expand immature human HSCs.

1.2.5 Thrombopoietin (TPO)

TPO is the ligand for Mpl and is responsible for the regulation of megakaryocyte proliferation and differentiation (Kaushansky *et al*, 1994). TPO has been shown to induce proliferation of primitive progenitors *in vitro* and has therefore become an important factor in *ex vivo* HSC expansion schemes (Kaushansky, 1997;Borge *et al*, 1997). The role of TPO in optimising HSC engraftment is reviewed recently by Verfaillie (Verfaillie, 2002b) and is discussed further in Chapter 5.

1.3 Isolation and characterisation of HSCs

1.3.1 Isolation and purification of HSCs

A variety of techniques have been used to enrich HSCs, including density centrifugation, activation and/or cell cycle status e.g. sensitivity to cell cycle-active cytotoxic agents (Orschell-Traycoff *et al*, 2000;Szilvassy & Cory, 1993), and surface antigen expression (Spangrude *et al*, 1988;Szilvassy *et al*, 1989;Baum *et al*, 1992), dye efflux properties (Goodell *et al*, 1996), but no unique characteristics have been found to identify these elusive cells specifically. An important point in the isolation of HSCs is the one-to-one correspondence between physically purified cells and their potential ability to function as stem cells.

1.3.1.1 Cell surface markers

Currently, there is no single phenotypic characteristic that can be used to define uniquely human or murine haemopoietic stem cells. However, using a variety of separation techniques and functional assays, numerous laboratories have established a pattern of surface antigen expression that can be used largely to isolate HSCs. The identification of rare populations highly enriched for stem cell (SRC and /or LTCIC) activity (see section 1.3.2) has come about due to the systematic functional analysis of HSCs

expressing a particular cell surface antigen or other markers. HSCs are characterised by the absence of lineage antigens expressed predominantly on terminally differentiated lymphocytes (CD45R/B220, CD3, CD4, CD8), myeloid (CD11b/Mac-1, Ly-6G/Gr-1), and erythroid cells (TER-119/molecule associated with cell surface Glycophorin A). Thus, many HSC isolation techniques use the removal of such lineage-positive cells to leave a suspension of predominantly immature cells.

1.3.1.2 CD34 as the universal marker of HSCs

Surface expression of the sialomucin CD34, a transmembrane cell surface glycoprotein, has rapidly become the distinguishing feature used as the basis for the enumeration, isolation, and manipulation of human stem cells, because CD34 is down regulated as cells differentiate into more abundant mature cells (Andrews *et al*, 1989;Krause *et al*, 1996). It was initially identified by the My10 mAb, which was raised against the immature myeloid cell line KG-1a (Civin *et al*, 1984). Other CD34 mAb include the clones HPCA2 (8G12) which is routinely used for detecting $CD34^+$ cells in cytokine MPB (Watts *et al*, 2002) and the antibody QBEnd10 used in $CD34^+$ cell selection in the commercial CD34 selection device from Miltenyi Biotec (Watts *et al*, 2002). The normal function of the CD34 molecule in haemopoiesis has remained enigmatic but a potential role in cell adhesion and in the homing process has been described (Healy *et al*, 1995;Lanza *et al*, 2001). CD34 is expressed selectively on stem cells and early progenitors during human (Civin *et al*, 1984;Berenson *et al*, 1991;Siena *et al*, 1991) and murine (Brown *et al*, 1991;Simmons *et al*, 1992) haemopoiesis, but both mouse and human CD34 are expressed outside the haemopoietic system on vascular endothelial cells (Fina *et al*, 1990) and some fibroblasts (Brown *et al*, 1991). Transplant studies in several species, including baboons (Berenson *et al*, 1988) and mice, have shown that long term marrow repopulation can be provided by $CD34^+$ cells (Horn *et al*, 2003). However, several recent studies have suggested that there may be human and murine stem cells that do not express CD34 (Zanjani *et al*, 1998;Bhatia *et al*, 1998;Goodell *et al*, 1997).

1.3.1.3 Other stem cell markers

In addition to the 'classical' phenotype, several novel markers of primitive HSCs have been identified over the past several years. These markers are summarised in Table 1.2, adapted from (Szilvassy, 2003). AC133 (CD133) is a transmembrane glycoprotein antigen of unknown function that is selectively expressed on the majority of $CD34^+$ cells from human BM, Foetal Liver and normal and mobilised peripheral blood (Yin *et al*, 1997). Details on CD133 expression in human stem cells are reviewed by (Bhatia, 2001). Several other markers have proven useful in further dividing the population into more functionally homogeneous populations e.g. current evidence indicates that all human HSCs are $CD38^-$, whereas more mature progenitor cells are $CD38^+$ (Peled *et al*, 1999b). Another novel marker allowing the identification of human HSCs is the vascular growth factor receptor 2 (VEGFR2), also known as KDR or Flk1 in mice (Ziegler *et al*, 1999). It had been reported that $CD34^+$ KDR^+ cells are highly enriched in putative HSCs (SRC and E-LTC-IC or CAFC) (Ziegler *et al*, 1999).

1.3.1.4 'Side population' of Dye efflux properties

A alternative method which exploits the ability of HSCs to efflux the fluorescent dye Hoechst has been used for obtaining enriched populations of HSCs from adult mouse bone marrow (Goodell *et al*, 1996). This strategy is based more on function than phenotype. The Hoechst 33342-low cells isolated were called side population (SP) cells and in 1997, SP cells were identified in adult bone marrow from several species, including humans (Goodell *et al*, 1997).

Table 1.2 - Phenotype of human and murine haemopoietic stem and progenitor cells, adapted from (Szilvassy, 2003).

Stem cells	Progenitor cells
Human	
$CD34^+$ and $^-$	$CD34^+$
$CD38^-$	$CD38^+$
Thy-1 ^{lo}	Thy-1^-
c-kit ^{lo}	c-kit^+
HLA-$DR^{-/lo}$	HLA-DR^+
$CD45RA^{-/lo}$	
$CD71^{-/lo}$	
$AC133^+$	
Rh-123^{lo}	Rh-123 ^{hi}
HO ^{lo}	HO ^{hi}
KDR^+ ($Flk1^+$)	$KDR^{-/lo}$
Adult Mouse BM	
Sca-1^+	Sca-$1^{-/+}$
$CD34^-$	$CD34^+$
$CD38^+$	CD38-/lo
c-kit ^{lo}	c-kit^+
Lin^-	Lin^-
Thy-1 ^{lo}	Thy-1^-
HO ^{lo}	HO ^{hi}
$Flk\text{-}2^-$	$Flk\text{-}2^+$

1.3.2 Characterisation of HSCs

Stem cells have an extensive ability to self-renew, and are able to persist in recipients for long periods of time after transplantation, continuously replenishing the blood with mature differentiated cells (Till & McCulloch, 1980). As repopulation assays cannot be carried out in humans, surrogate *in vitro* and *in vivo* assays are used to evaluate HSCs.

1.3.2.1 Surrogate in vitro assays

Quantitative analyses of human HSPC have historically been limited to *in vitro* assays where the proliferative potential of stem cells is evaluated in the presence of various combinations of cytokines. *In vitro* assays used to assess primitive human progenitors include several stromal based assays including the long term culture initiating cell (LTC-IC) assays, cobblestone area-forming cell (CAFC) assays and extended LTC-IC (E-LTC-IC) assays

(reviewed in (Gordon, 1993)). The LTC-IC assay detects primitive cells capable of giving rise to colony forming cells (CFCs) after 5-8 weeks of culture on a stromal cell (feeder) layer (Sutherland *et al*, 1990;Sutherland *et al*, 1989). The E-LTC-IC assay is a modification of the LTC-IC assay where there is an extended period preculture, up to 10 weeks (Hao *et al*, 1996). This E-LTC-IC assay detects even more primitive cells than week 5 or week 8 LTC-IC (Hao *et al*, 1996). Both the LTC-IC and the E-LTC-IC assays are time consuming and cumbersome to perform. The CAFC assay is used to assess the frequency of primitive HSCs subsets (Ploemacher *et al*, 1989;Breems *et al*, 1994). This assay scores a particular morphologic growth pattern, which forms a cobblestone appearance in the stromal monolayer. The time required for generation of these colonies defines the CAFC assay. Appearance of cobblestones within 7 days indicates progenitor cells, while appearance after several weeks to 1 month indicates a more primitive stem cell (Neben *et al*, 1993).

The most common approaches to quantify multi-lineage or single lineage committed haemopoietic progenitors, called colony-forming cells (CFCs) or colony forming units (CFUs), utilise viscous or semi-solid matrices and culture supplements that promote their proliferation and differentiation and allow the clonal progeny of a single progenitor cell to stay together and thus form a colony of mature cells (Gordon, 1993). In such assays, committed cells, which can give rise to cells of single lineages are named after the cell type of the colonies obtained (for instance colony forming cells-granulocyte). In addition, bipotent cells giving rise to colonies with cells of different lineages can be detected e.g. GM-CFC. The disadvantage of the assay is that it is critically dependent on the culture conditions used, including the type of serum and the cytokines used for stimulation. However, the availability of commercially available standardised reagents has led to increased reproducibility of CFC assays. CFC assays are used to quantify and characterise haemopoietic progenitors from different sources (e.g. BM, UCB, MPB) and for quality control of clinical stem cell collection, processing and cryopreservation. They are also used to investigate progenitor responses to growth factors, to quantitate progenitor cell numbers after *ex vivo* expansion.

1.3.2.2 *In vivo assays*

A conclusive way to assay stem cells is based on their capacity to repopulate the entire haemopoietic system in conditioned recipients after transplantation. In an attempt to develop *in vivo* animal models for human haemopoiesis several groups have transplanted human cells in xenogeneic transplant recipients e.g. immunodeficient mice (McCune, 1996;Dick, 1996) and foetal sheep (Zanjani *et al*, 1997).

1.3.2.3 NOD/SCID mouse model

For the purpose of studying HSPC transplantation, several animal models have been developed, including the severe combined immuno-deficient (SCID) mice (Bosma *et al*, 1983), severe combined immuno-deficient-human (SCID-hu, foetal bone fragments are implanted under the skin; (McCune *et al*, 1988)), non-obese diabetic/severe combined immuno-deficient (Shultz *et al*, 1995) (NOD/SCID, transplanted cells need to migrate actively to the marrow) and non-obese diabetic/severe combined immuno-deficient β2 microglobulin (NOD/SCID β2m, reduced innate immunity in comparison to NOD/SCID mice) models (reviewed in (Voermans *et al*, 2001;Dao & Nolta, 1999;Greiner *et al*, 1998)).

The SCID mice, homozygous for the SCID mutation lack both humoral and cell-mediated immunity due to the absence of mature T or B lymphocytes (Bosma & Carroll, 1991). The lack of an adaptive immune system in SCID mice results from their inability to express rearranged antigen receptors (Lieber *et al*, 1988;Malynn *et al*, 1988). This defect is due to a defective recombinase activity (a failure to activate a DNA recombinase enzyme) that results in abnormal and non-functional antigen receptor gene rearrangements (Bosma & Carroll, 1991). The arrest in lymphocyte development is not absolute as some young adult SCID mice are 'leaky' and generate a few clones of functional B and T cells (Nonoyama *et al*, 1993;Shultz *et al*, 1995;Bosma *et al*, 1988). SCID mice that have serum Ig levels greater than 1μg/ml are considered 'leaky' and by 10-14 months of age, virtually all SCID mice are 'leaky'. Most of the earlier studies used the

SCID mice, which required high cell doses to overcome significant antigen non-specific immunity.

The creation of the NOD/SCID mice by crossing the SCID gene onto NOD background, proved to be a better recipient because this mice strain appeared to have lower NK and complement activities, and a defect in macrophages (Shultz *et al*, 1995). Ten to twenty fold lower number of cells were necessary to engraft NOD/SCID β2m ², when compared with NOD/SCID mice (Glimm *et al*, 2001). The NOD/SCID β2m mice has become available recently and is thought to be a even better recipient than the NOD/SCID mice because of reduced innate immunity (lack NK cells) in comparison to NOD/SCID mice (Kollet *et al*, 2000).

1.3.2.4 The foetal sheep HSC assay

This is a large animal model of human haemopoiesis based on the permissive environment of the early gestational age foetus of a sheep (Zanjani *et al*, 1996). This assay allows the long term engraftment and multi-lineage expression of human HSCs in the absence of irradiation or other myeloablative therapies (Zanjani *et al*, 1992;Srour *et al*, 1992). Due to the high costs of this model it is not utilised widely.

1.4 Sources of HSCs

HSCs have been collected from adult bone marrow, mobilised peripheral blood and umbilical cord blood (Korbling & Anderlini, 2001). Primitive HSCs are normally present in very low numbers in peripheral blood (Barr *et al*, 1975;Hong & Deeg, 1994). However the numbers of progenitor cells in peripheral blood can be greatly increased (mobilised) after treatment with chemotherapy and/or cytokines e.g. G-CSF, the most widely used cytokine for mobilisation of stem cells (Watts *et al*, 2000). Mobilised peripheral blood is an attractive stem cell source for clinical use. HSCs obtained from umbilical cord blood are numerically fewer (rarely able to extract more than a few million from one donor) but on a per cell basis, UCB cells engraft 10 to 15 fold better in xenogeneic hosts than BM progenitors (van der Loo *et al*, 1998a;Holyoake *et al*, 1999). HSCs from G-CSF mobilised PB grafts (MPB)

have become the preferred cell source for transplantation because of earlier neutrophil and platelet recovery, which is though to be due to the increased number of STRCs (Korbling & Anderlini, 2001). HSCs found in MPB may be qualitatively different from those found in the BM and evidence for this comes from a study which compared BM $CD34^+$ Lin^- cells with PB $CD34^+$ Lin^- cells. These workers found that the proliferation and differentiation capacity inferior in PB $CD34^+$ Lin^- cells (Prosper *et al*, 1996). One study in a xenogeneic transplant model found that more MPB than BM cells are required to reconstitute haemopoiesis (Verfaillie *et al*, 2000;van der Loo *et al*, 1998a), and the long-term repopulation ability of peripheral blood HSPCs, as assessed by secondary and tertiary repopulation assays, may be inferior to that of BM progenitors (Verfaillie *et al*, 2000;van der Loo *et al*, 1998a). Another important source of HSCs in research, but not in clinical use, is from foetal blood and liver (Yong *et al*, 2002a).

1.5 Clinical applications of HSC transplantation

Haemopoietic stem cell transplantation is performed to rescue a patient's haemopoietic system after myeloblative chemo- or radiotherapy. In these applications, the patient's own cancerous haemopoietic cells are destroyed via radiation or chemotherapy, then replaced with a bone marrow transplant, or, as is done now, with a transplant of HSCs collected from the peripheral circulation of a matched donor. In the case of autologous stem cell transplantation, HSCs are collected from mobilised peripheral blood and then cells are frozen and stored while the patient undergoes intensive chemotherapy or radiotherapy to destroy the cancer cells at the same time ablating the haemopoietic system. The HSCs are then re-infused into the patient to reconstitute haemopoiesis. HSC transplantation is in widespread use for the treatment of haematological malignancies (Korbling & Anderlini, 2001). Haematological malignancies include ALL, AML, CML, Hodgkin's disease, multiple myeloma, and non-Hodgkin's lymphoma. Non malignant disorders are also treated with BMT/HSCT e.g. aplastic anaemia and SCID.

1.6 Biological basis of HSC transplantation

HSPCs migrate between different tissues in foetal life and continue to recirculate in adult life. During development, HSCs initially migrate from the AGM (aortogonadomesonephros) (Godin *et al*, 1995;Medvinsky & Dzierzak, 1996) to the foetal liver, and later, to the spleen and bone marrow. In adults, small numbers of stem cells circulate in the bloodstream (Barr *et al*, 1975;Hong & Deeg, 1994), and this number can be augmented by chemotherapy (To *et al*, 1984;Tavassoli, 1992) and growth factors and is referred as 'mobilisation' (Watts *et al*, 2000). Thus, these cells must be able to selectively localise in haemopoietic tissue.

A special feature of intravenously transplanted haemopoietic stem and progenitor cells is their ability to migrate from the peripheral blood to the bone marrow, a process referred to as 'homing' (Papayannopoulou, 2003). The ability of circulating HPCs to home to the extravascular compartment of the bone marrow is of central importance to the clinical practice of HPC transplantation. The reverse process, by which HPCs are 'mobilised' from the marrow into the general circulation, is now exploited clinically to obtain circulating HPCs for autologous and allogeneic transplantation. Despite this clinical and biological significance, the mechanisms by which the process of homing of HPC to the bone marrow microenvironment are mediated are still not fully understood.

1.7 Functional properties of HSC

1.7.1 Homing of HSC to the Bone Marrow

1.7.1.1 Definition of homing

Confusion regarding the definition of homing still exists in the field because, in most instances, homing is equated with engraftment (see section 1.7.4). Recently, however more attention has been focussed on the initial homing phases of transplantation. "Homing" refers to the selective ability of circulating HSPC to localise within the bone marrow (BM), and involves a complex series of adhesive and migratory processes which are still not fully understood (reviewed in (Papayannopoulou, 2003;Quesenberry & Becker,

1998;Hardy, 1995;Tavassoli & Hardy, 1990;Hardy & Megason, 1996)). It is generally considered to be a multi step process analogous to the mechanism of leukocyte extravasation (von Andrian *et al*, 1991;Imhof & Dunon, 1995). In this model, cells initially roll on vascular endothelium tethered by selectins and their respective ligands. Cell activation by signalling molecules, such as the chemokines, which are released by vascular and non vascular cells, leads to upregulation of integrin function, and the high affinity binding of activated integrins to endothelial ligands produces tight adhesion, shape change, and diapedesis to localise in extravascular foci (Imhof & Dunon, 1995), Figure 1.2 (Adapted from (Peled *et al*, 2000)).

Studies on homing of HSC to the BM have also pointed to a multi step process mediated by a variety of adhesion molecules and receptors (reviewed in (Voermans *et al*, 2001;Quesenberry & Becker, 1998;Papayannopoulou & Craddock, 1997)), including E, and P selectin (CD62E and CD62P), VLA-4 (CD49d/CD29), VLA-5 (CD49e/CD29), LFA-1 (CD11a/CD18), and the stromal derived factor 1 (SDF-1) receptor CXCR4 (Williams *et al*, 1991;Papayannopoulou *et al*, 1995;van der Loo *et al*, 1998b;Aiuti *et al*, 1997;Frenette *et al*, 1998;Mazo *et al*, 1998;Peled *et al*, 1999b;Peled *et al*, 1999a). The selectins (E and P selectins) mediate initial engagement of rolling cells and tethering of $CD34^+$ cells with BM sinusoidal endothelium (Frenette *et al*, 1998). The integrins (VLA-4 and VLA-5) are considered to be important for anchorage and migration into the BM tissue (Papayannopoulou *et al*, 2001). For example, a blocking antibody to VLA-4 or its ligand (VCAM-1), has been shown to reduce the accumulation of progenitors cells in the BM 3hr after transplantation (Papayannopoulou *et al*, 1995). The chemoattractant SDF-1 (ligand to the receptor CXCR4) which is released by BM stromal cells leads to upregulation of integrin function, and the high affinity binding of activated integrins to endothelial ligands and tight adhesion (Hidalgo *et al*, 2001;Wright *et al*, 2002;Peled *et al*, 2000;Peled *et al*, 1999a), Figure 1.2 adapted from (Peled *et al*, 2000).

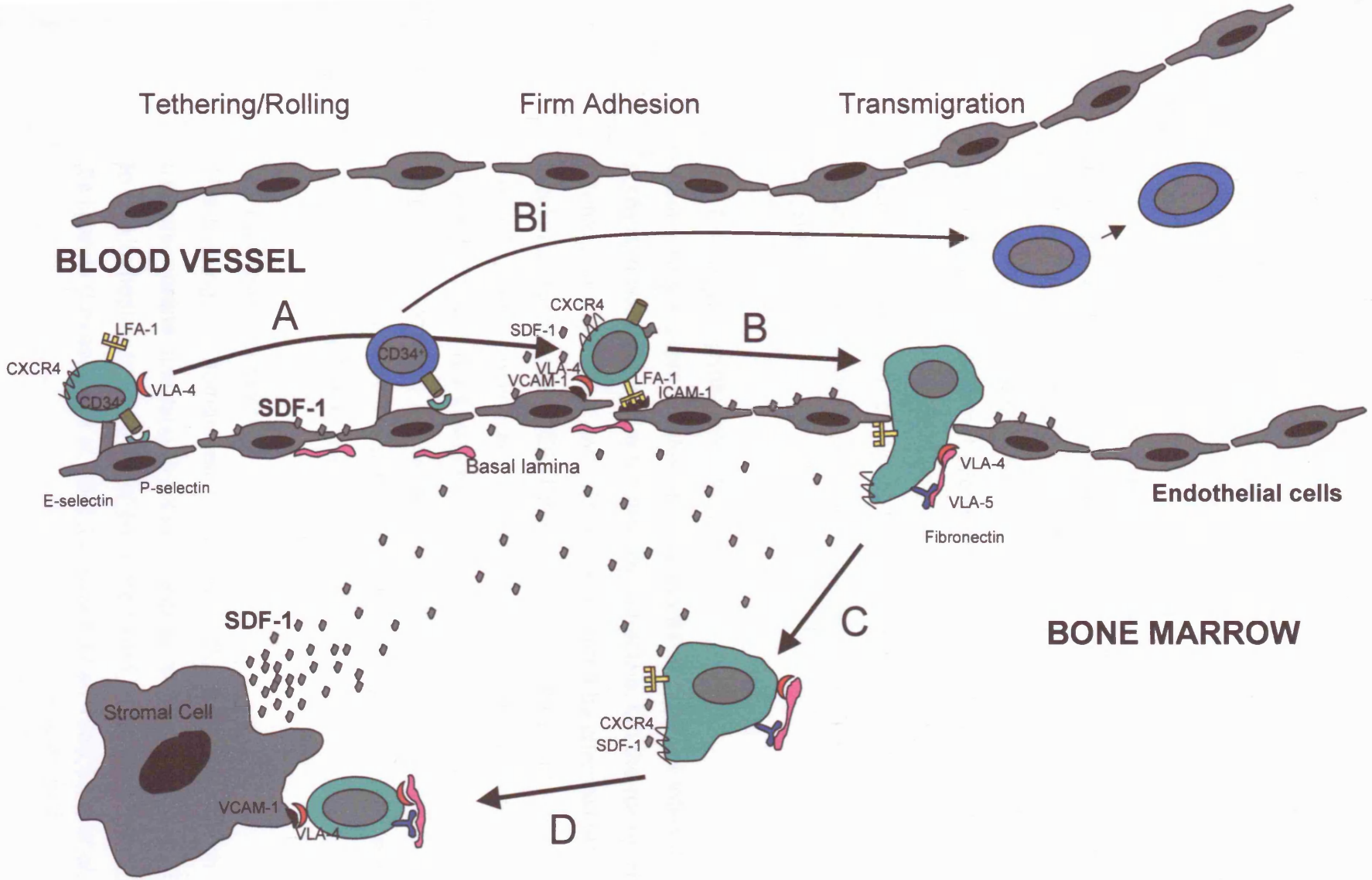


Figure 1.2 - HSPC rolling, firm adhesion and transendothelial migration. (A) HSC rolling interactions on constitutively expressed E and P selectins. Following rolling, CXCR4$^+$ stem cells are activated by SDF-1, which is secreted from BM endothelial cells and triggers LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions promoting firm adhesion to endothelial cells (B). Cells that do not express sufficient levels of CXCR4 will detach from the endothelial layer and return to the blood stream (Bi). (C) The arrested human CXCR4$^+$ HSC, in response to SDF-1, will extravasate and migrate through the underlying basal lamina ECM using VLA-4 and VLA-5 integrin receptors to fibronectin. (D) Migrating stem cells will eventually reach the 'stem cell niches', which consist of stromal cells that present the proper set of adhesion molecules (e.g. VCAM-1 and Fibronectin), SDF-1, and growth stimulatory factors (Modified from Peled *et al.*, 2000).

1.7.1.2 Model of HSC homing

1.7.1.3 Tethering/Rolling

The first step in the migration consists of tethering and rolling of HPC along the endothelium (Zannettino *et al*, 1995;Dercksen *et al*, 1995;Schweitzer *et al*, 1997). Tethering refers to the establishment of loose and somewhat transient adhesion between HPCs and endothelium (Figure 1.2; A). Because of the shear forces by the blood stream, this results in the rolling of HPCs along the endothelium. It is the selectins (E-selectin, P-selectin and L-selectins) and their ligands that mediate this tethering/rolling step (Hardy, 1995;Hardy & Megason, 1996;Tavassoli & Hardy, 1990;Quesenberry & Becker, 1998;Frenette *et al*, 1998;Papayannopoulou *et al*, 2001), a short-term cell-cell contact that allows HPCs to sample the local endothelium for the presence of specific trigger factors that can activate HPC integrins and allow the cascade to continue. In the absence of such factors, the transient nature of selectin binding allows HPCs to disengage and move on (Figure 1.2; Bi).

1.7.1.4 Triggering/Integrin activation

Rolling HPCs express substantial amounts of different integrins on the cell surface, however they are functionally not active. On the rolling HPC, β1 and β2-integrins e.g. VLA-4 and LFA-1 are activated by chemokines (chemotactic cytokines) for example SDF-1 the major chemoattractant for HPC (Prosper & Verfaillie, 2001), which are located on the endothelial surface or bound to proteoglycans in the endothelial extracellular matrix. The activation of HPCs triggers an increase in avidity caused by a conformational change in the integrin heterodimer, resulting in greater affinity for ligands and/or to post-receptor events (Ginsberg *et al*, 1992).

1.7.1.5 Strong adhesion

The binding of chemokines to their specific receptors, which are seven-transmembrane spanning G-protein coupled receptors (Rollins, 1997), leads to firm adhesion to endothelial cells mediated by the binding of integrins to it's ligands (Levesque *et al*, 1995;Levesque *et al*, 1996;Sato *et al*, 2000). The

predominant pathways are mediated by the integrin adhesion molecule pairs VLA-4/VCAM-1 and LFA-1/ICAM-1 (Papayannopoulou, 2003;Papayannopoulou & Craddock, 1997;Papayannopoulou *et al*, 1995;Papayannopoulou *et al*, 2001).

1.7.1.6 Transmigration

The firm adhesion is followed by transendothelial migration of HPC (Mohle *et al*, 1999;Mohle *et al*, 2001). The process of transmigration starts with locomotion of adherent HPCs toward the endothelial cell-cell junction. This is a rapid event and is a one-way trafficking event resulting the cell reaching the subendothelial basal membrane. Prior to migration in response to a chemotactic stimulus, leukocytes (and probably HPC) display a complex repertoire of motility associated changes, including shape change, polarisation of signalling complexes, actin polymerisation, and polarised adhesion. Co-ordinated formation of lamellipodia and acto-myosin-based contractility moves the cell forward, in the direction of the chemotactic gradient (Figure 1.2; B). Recently the transmigration of $CD34^+$ cells across BMECs and HUVECs has been demonstrated to be mediated by PECAM-1 (CD31) (Yong *et al*, 1998). PECAM-1 is an Immunoglobulin superfamily cell adhesion molecule that is concentrated at the junctions between endothelial cells and is also expressed on platelets, monocytes, neutrophils and a subset of T cells. Muller *et al.,* (1993) demonstrated that PECAM-1 was also directly involved in the process of monocyte diapedesis between endothelial cells (Muller *et al*, 1993).

Finally interaction between adhesion molecules on HPC and ligands in the stroma leads to anchoring of the HPC in the bone marrow microenvironment (Aiuti *et al*, 1997;Yong *et al*, 1998;Mohle *et al*, 1997). The anchoring of HPC depends mainly on adhesion via integrins to stromal cells and to the extracellular matrix proteins, Figure 1.2; C and D (Turner *et al*, 1998;Kerst *et al*, 1993;van der Schoot & Dercksen, 1994).

1.7.2 Adhesion molecules and receptors involved in HPCs homing

1.7.2.1 Selectins and their ligands

The selectins (reviewed in (Gonzalez-Amaro & Sanchez-Madrid, 1999;Chan & Watt, 2001;Imhof & Dunon, 1995)) are a family of three proteins, E (endothelial, CD62E), P (platelet, CD62P) and L (Leukocyte, CD62L)-selectins. All the selectins structurally are composed of a 120 amino terminal C-type calcium-dependent domain, followed by an epidermal growth factor-like domain and two to nine short consensus repeat units homologous to domains found in complement binding proteins. E-selectin and P-selectin are expressed by endothelial cells following stimulation by inflammatory mediators (e.g. IL-1β, TNF-α and INF-γ), P-selectin is also present on activated platelets and L-selectin is constitutively on leukocytes, including $CD34^+$ cells. The role of selectins in the homing process has been studied in genetically deficient mice and with antifunctional antibodies. These knockout mice show defects in HPC homing to the bone marrow, but not the spleen, after bone marrow transplantation into irradiated recipients (Frenette *et al*, 1998). Although homing was not influenced when neutralising antibodies against a single selectin were used, homing studies in mice deficient in both E and P selectins showed that E/P selectins and α4 integrins contributed equally to this process (Mazo *et al*, 1998;Frenette *et al*, 1998). Also irradiated E/P selectin-deficient mice in transplantation experiments had early deaths (Frenette *et al*, 1998). All 3 selectins can bind to sialylated, fucosylated lactosamines, sialyl Lewis x, and sialyl Lewis a, when presented on the appropriate backbone and L-selectin bind to CD34.

1.7.2.2 Integrins and their ligands

Integrins are adhesion molecules that are heterodimeric proteins consisting of noncovalently linked α and β subunits. At present at least 17 α subunits (α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_V, α_L, α_M, α_X, α_{iib}, $\alpha_{iE,}$ α_H and α_D) and 8 β subunits (β1, β2, β3, β4, β5, β6, β7 and β8) exist and as many as 24 different integrin combinations have been reported, while many α subunits associate with a single β subunit, several α subunits can associate with more than one type of β subunit (reviewed in (Gonzalez-Amaro & Sanchez-Madrid,

1999;Imhof & Dunon, 1995;Reddy & Mangale, 2003)). The integrin subunit association and classification is outlined in Table 1.3. Both VLA-4 and VLA-5 β1 integrin receptors play roles in haemopoietic development and migration (Papayannopoulou & Nakamoto, 1993;Papayannopoulou *et al*, 1995). For example, a blocking antibody to VLA-4 or its ligand (VCAM-1), has been shown to reduce the accumulation of progenitors cells in the BM 3hr after transplantation (Papayannopoulou *et al*, 1995).

Table 1.3 - Integrin classification (adapted from(Reddy & Mangale, 2003))

Family	β subunit	α subunit	αβ complex	Receptor	Ligand
VLA proteins	β1	α1	α1β1	VLA-1	
		α2	α2β1	VLA-2	L,C
		α3	α3β1	VLA-3	C,Fn,entactin
		α4	α4β1	VLA-4	Fn,VCAM-1
		α5	α5β1	VLA-5	Fn
		α6	α6β1	VLA-6	L
		α7	α7β1		Fn, Vn, Tn
		α8	α8β1		Tn, Fn, Vn
		α9	α9β1		
		αv	αvβ1		
Leukocytes proteins	β2	αL	αLβ2	LFA-1	ICAM-1,2,3
		αM	αMβ2	MAC-1	C3b,Fb, ICAM-1
		αX	αXβ2	P150, 95	C3b, LPS
		αD	αDβ2		ICAM-3
	β7	α4	α4β7		
		αH	αHβ7	LPAM-1	Fn, VCAM-1
		αE	αEβ7		E-cadherin
Cytoadhesin	β3	αV	αVβ3	Vitronectin	Vn,Fb, Fn,OPN, VWF, Tn, Thr
		αiib	αiibβ3	GpIIb/IIIa	Fb, Fn, VWF, Vn
Other combinations	β5	αV	αVβ5		Vn, Fn
	β6	αV	αVβ6		Fn, Tn
	β8	αV	αVβ8		Vn
	β4	α6	α6β4		L

C=collagen, Fn=fibronectin, L=laminin, M=macrophage, Tn=tenasin, Fb=fibronogen, Vn=vitronectin, VWF=von willebrand factor, Thr=thrombospondin, OPN=osteopontin, LFA=leukocyte function associated antigen, VLA=very late antigen

1.7.2.3 Chemokines

Chemokines (chemotactic cytokines) are a group of small (8-14kDa) molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G-protein-coupled receptors (Zlotnik & Yoshie, 2000;Baggiolini *et al*, 1997). Chemokines induce the upregulation and activation of integrins, which enable the HPCs to adhere to the endothelial cells to the vessel wall before migrating into the tissue (Springer, 1994).

Chemokines have been divided into two major subfamilies (α and β chemokines) on the basis of the arrangement of the two amino terminal cysteine residues, depending on whether the first two cysteine residues have an amino acid between them (CXC) or adjacent (CC) (Rollins, 1997;Zlotnik & Yoshie, 2000). The receptors for these chemokines are also divided into subfamilies, i.e. the CXCR, CCR, CR and CX3CR family (Zlotnik & Yoshie, 2000).

In 1997, SDF-1 was described as the first powerful chemoattractant for $CD34^+$ cells. SDF-1 is produced by stromal cells, including those from the BM (Aiuti *et al*, 1997;Mohle *et al*, 1998). SDF-1 is classified as a CXC-chemokine and is also a chemoattractant for monocytes and lymphocytes (Bleul *et al*, 1996). The receptor for SDF-1 is a G-protein coupled receptor called CXCR4 (also known as Fusin and LESTER). The chemokine SDF-1 and its receptor CXCR4 have been implicated in selectively directing the homing of HSCs to the BM (reviewed in (Lapidot & Kollet, 2002;Lapidot & Petit, 2002)). The engraftment of human cells in NOD/SCID mice was prevented by treatment with antibodies against CXCR4 (Peled *et al*, 1999b). The expression of CXCR4 on $CD34^+$ human HSCs has suggested a role for SDF-1 in the homing process (Peled *et al*, 1999b). Peled *et al.,* (1999b) reported that surface CXCR4 levels on freshly isolated human BM or CB $CD34^+$ enriched cells are fairly constant (about 40-50%), as is their migratory ability toward SDF-1 in transwells (about 25%). However, MPB $CD34^+$ enriched cells obtained from G-CSF-treated healthy donors display highly variable expression of CXCR4, and migration toward a gradient of SDF-1 (Peled *et al*, 1999b). Aiuti *et al.,* (1997) found very low levels of surface CXCR4

expression on mobilised human $CD34^+$ enriched cells and reduced migration *in vitro* toward a gradient of SDF-1 in transwells (Aiuti *et al*, 1997). However, Rosu-Myles *et al.,* (2000) recently demonstrated that CXCR4 expression on human HSCs was not required for effective stem cell repopulation function (Rosu-Myles *et al*, 2000). They reported that human CB and foetal blood $CD34^+$ $CD38^-$ $CXCR4^-$ and $CD34^+$ $CD38^-$ $CXCR4^+$ sorted cells both have similar repopulating capacity in NOD/SCID mice (Rosu-Myles *et al*, 2000). These observations by Rosu-Myles *et al.,* (2000) may be because despite the absence of CXCR4 on the surface, $CD34^+$ $CXCR4^-$ sorted cells express intracellular CXCR4 that can be induced to cell surface expression by stimulation with cytokines (Kollet *et al*, 2002). This suggests that perhaps CXCR4 levels may be modulated *in vivo* (Kollet *et al*, 2002).

1.7.3 Cell cycle status of HSCs from different sources

The majority of haemopoietic stem cells are non-cycling quiescent cells, but with the potential of multiple divisions with self-renewal capacity as well as giving rise to different committed maturing cells. Adult human MPB show a highly quiescent phenotype (Uchida *et al*, 1997) with greater than 95% of peripheral blood $CD34^+$ cells in G_0/G_1 of the cell cycle (Yong *et al*, 1998;Williams *et al*, 1997). Similarly more than 97% of UCB $CD34^+$ cells are in G_0/G_1 (Traycoff *et al*, 1994;Yong *et al*, 1999). In contrast only 85% of adult BM $CD34^+$ cells are in the G_0/G_1 phase of the cell cycle (Gothot *et al*, 1998). HSC found in extravascular haemopoietic tissue appear to contain a higher proportion of cells in the proliferative phase ($S+G_2/M$) of the cell cycle. Thus, while foetal $CD34^+$ cells are in general less quiescent than adult $CD34^+$ cells, those from foetal liver contain a greater proportion of cells in $S+G_2/M$ (28%) than those found in foetal blood (11%) (Yong *et al*, 2002a).

1.7.4 Engraftment of HSCs

Following transplantation into the recipient, HSCs, in order to engraft and reconstitute the BM, must home to and lodge in the specialised niches of the BM microenvironment (see section 1.7.1) and this is followed by the onset of their proliferation (engraftment). One of the factors that may be involved in engraftment ability of HSCs is the homing process itself, as discussed above

(see section 1.7.1) and also in more detail in Chapter 3. The other factors including cell cycle status (Chapter 4), cytokine exposure (Chapter 3 and 5), CXCR4 expression (Chapter 3), Fas expression (Chapter 5) and accessory cells (Chapter 6) and are discussed in detail in the chapters indicated and highlighted below.

1.7.4.1 Factors influencing engraftment ability of haemopoietic stem cell grafts

1.7.4.2 Cytokine exposure

One of the major efforts in stem cell research has been on *ex vivo* expansion of HSPCs. Cytokine expansion may, however have detrimental effects on subsequent engraftment (Liu *et al*, 2003;Young *et al*, 2001). The effect of cytokine exposure on HSPCs has been demonstrated in both murine and xenogeneic systems and studies highlighting that cytokine exposure alters engraftment potential of HSCs is discussed in detail in Chapters 3 and 5. The reasons for this remains unclear, but are commonly thought to relate to cell cycling.

1.7.4.3 Cell cycle status

One of the major factors is thought to be the role of the cell cycle status in homing of HPCs. This has been shown in both syngeneic murine models (Habibian *et al*, 1998;Fleming *et al*, 1993) and also in xenogeneic transplant models (Gothot *et al*, 1998;Glimm *et al*, 2000). Studies in the xenogeneic transplant model observed that human haemopoietic stem cells lose engraftment potential during their $S/G_2/M$ transit. This is discussed in detail in Chapter 4 and also studies modulating the cell cycle progression and it's effects on engraftment.

1.7.4.4 CXCR4 expression

Studies by Peled *et al.,* (1999b) showed the influence of the CXCR4 receptor in affecting the ability of HSC to engraft (Peled *et al*, 1999b). This group demonstrated in the murine NOD/SCID model that the engraftment of human HSCs can be impaired by antibodies against CXCR4 (Peled *et al*, 1999b). In the clinical setting, Spencer *et al.,* (2001), enumerated CXCR4-positive HSC

(MPB) used for allogeneic transplantation and investigated the relationship with rate of subsequent haemopoietic reconstitution (Spencer *et al*, 2001). This group reported that transplantation of a minimum of $2.5x10^6$ CXCR4 CD34 positive cells/kg ensured rapid post-transplant platelet recovery. They concluded that co-expression of CXCR4 on CD34-positive cells may be an important determinant of post-transplant engraftment (Spencer *et al*, 2001). The influence of CXCR4 in engraftment ability of HSC grafts is discussed in detail in Chapters 1, 3 and 5.

1.7.4.5 Fas (CD95) expression

Ex vivo expansion is associated with increased expression of Fas (CD95) on cultured HSCs (Young *et al*, 2001;Liu *et al*, 2003). This increased Fas expression could make these HSCs more susceptible to apoptosis upon transplantation into recipients and in turn influence the engraftment ability of the expanded HSC graft. This is discussed in detail in Chapter 5.

1.7.4.6 Accessory cells

Recent results have pointed to the enhancing effect on engraftment ability of HSC grafts by co-transplantation in the presence of accessory cells. Examples of these accessory cells include *ex vivo* expanded MSCs and $CD8^+$ T cells, discussed in detail in Chapter 6.

1.8 Other adult stem cells found in the BM (non-haemopoietic)

Aside from HSCs, BM also contains mesenchymal stem cells (MSCs) (Pittenger *et al*, 1999) and the recently identified mesodermal progenitor cells (MPCs) (Reyes *et al*, 2001). These two types of marrow derived stem cells are outlined below and discussed in detail in Chapter 6.

1.8.1 Types of marrow-derived stem cells

The bone marrow was originally thought to function mainly as a structural framework for the haemopoietic stem and progenitor cells (HSPC; discussed in detail in Chapters 3, 4, and 5) in the bone marrow. Since then, it has been established that the stroma consists of a heterogenous population of cells including endothelial, fibroblasts, adipocytes, and osteogenic cells, a subset

of which exerts both positive and negative regulatory effects on the proliferation and differentiation of haemopoietic cells (Dexter *et al*, 1977;Verfaillie, 1993). The adherent stromal cell population is also believed to contain other non-haemopoietic cells that are capable of self-renewal and differentiation into bone, cartilage, muscle, tendon and fat (Bruder *et al*, 1997;Mackay *et al*, 1998;Pittenger *et al*, 1999). These characteristics have led to many workers to refer to these cultured cells as Mesenchymal Stem Cells (MSCs) reviewed by (Short *et al*, 2003). Recently a second rare cell type which is termed Multipotent Adult Progenitor Cells (MAPCs) was identified by (Reyes *et al*, 2001).

1.8.2 Mesenchymal stem cells (MSCs)

The MSCs were first identified by Fridenshtein (Fridenshtein, 1982), who demonstrated that when BM is plated in FCS containing medium, colonies of adherent fibroblast-like cells develop that can differentiate to form osteocytes, chondrocytes, adipocytes, tenocytes and BM stromal fibroblasts under defined *in vitro* conditions (Pittenger *et al*, 1999;Deans & Moseley, 2000). MSCs can be purified on the basis of their ability to adhere to plastic or using mAbs SH2 and SH4 or Stro-1 (Gronthos *et al*, 1994;Pittenger *et al*, 1999). While there is not a clearly defined cell surface antigenic phenotype for MSCs, there is agreement that they do not express typical haemopoietic cell surface antigens such as CD45 and CD34 and therefore have been characterised phenotypically in humans as non-haemopoietic cells. Human MSCs can be identified flow cytometrically by the mAbs SH2 (CD105), SH3, and SH4 (CD73) (Pittenger *et al*, 1999). Some of the other surface antigens reported to be on these cells are CD13, CD49e and CD49b, β1 integrins, CD44, CD71, CD90, CD106 (VCAM-1), CD124 (IL-4 receptor) (Pittenger *et al*, 1999). Variation in the isolation techniques and culture media used to grow MSCs in different laboratories has led to variable findings regarding the differentiation potential of these cells. For example, (Reyes *et al*, 2001) report that there was no evidence that MSCs cultured under conditions initially developed by (Pittenger *et al*, 1999) differentiate into cells with endothelial characteristics (Reyes *et al*, 2001). However, very recently Oswald *et al.,* (2004) have shown that culture of confluent MSCs in low-serum (2% FCS)

supplemented with VEGF (50ng/ml), results in the acquisition of several features of mature endothelium, including the expression of VEGF receptors (KDR and FLT1), VE-cadherin, VCAM-1, and vWF. They show also an enhanced ability to form capillary-like structures in semi-solid medium (Oswald *et al*, 2004). As another example of the controversy regarding the differentiative capacity of MSCs, some workers report that MSCs can be differentiated into neuronal-type cells, whereas others have not succeeded in differentiating MSCs into neuronal cells (Azizi *et al*, 1998).

1.8.3 Multipotent adult progenitor cells (MAPCs)

A population of highly plastic, adult–derived BM cells, referred to as multipotent adult progenitor cells (MAPCs), can be grown *in vitro* from postnatal marrow (and other organs; (Jiang *et al*, 2002b)) of mice, rats and humans (Jiang *et al*, 2002a). The isolation and *ex vivo* expansion of MAPCs from adult derived-BM cells was first described by (Reyes *et al*, 2001), who demonstrated that these cells can differentiate at the single cell level not only into MSCs, but also into cells of visceral mesodermal origin, such as endothelium (Reyes *et al*, 2001;Reyes *et al*, 2002). They co-purify initially with MSCs and grow as adherent cells *in vitro*. MAPCs were selected by depleting BMMNCs of $CD45^+$ and $GlyA^+$ cells and culture on fibronectin with EGF and PDGF-BB and 2% or less FBS (Reyes *et al*, 2001). MPCs phenotypically are negative for CD34, CD45, c-Kit, HLA Class1 and HLA-DR. Cells expressed low levels of β2microglobulin, CD44 and high levels of CD13 and CD49b (Reyes *et al*, 2001). However unlike MSCs, MAPCs can be cultured indefinitely and expanded in culture for more than 80 population doublings (Reyes *et al*, 2002).

1.9 PhD Aims

To fully explore the clinical potential of HSCs it is important to understand the molecular basis for the observed loss of repopulating stem cells resulting from *ex vivo* manipulation and to investigate ways of improving the efficacy of HSPC transplantation by modulating the ability of stem cells to home to the BM.

The initial aims of the thesis were to characterise the changes in engraftment behaviour in conjunction with alteration in adhesion molecule expression and homing, of MPB $CD34^+$ cells after culture with SCF, Flt3L, IL-3 and IL-6. We quantified short term homing of human CFC to different organs and of PKH26 labelled $CD34^+$ cells homing to the BM in NOD/SCID animals in parallel with engraftment studies carried out on the same cells. I have investigated whether the reduced engraftment of cytokine stimulated $CD34^+$ cells is directly related to an alteration in short term organ specific homing (Chapter 3).

The second aim of the thesis was to determine if alterations in the homing and engraftment of cytokine stimulated MPB $CD34^+$ cells are cell cycle dependent. I investigated the cell cycle dependence of the homing and engraftment process by using sorted cells in G_0G_1 and $S/G_2/M$ phases of the cell cycle, and also by inducing cell cycle arrest at the G_1/S border, followed by synchronised S phase progression (Chapter 4).

The third aim of the thesis was to test several *ex vivo* manoeuvres (culture condition and blockade of Fas L) which have been reported to restore the engraftment of preactivated cells, and to investigate if such strategies were able to modulate the homing behaviour of adult $CD34^+$ cells (Chapter 5).

The final aim of the thesis was firstly, to isolate, expand and characterise MAPCs from postnatal BM and secondly to determine if co-transplantation of non-haemopoietic stem cells and heterologous T cells enhanced $CD34^+$ cell homing to the BM in the NOD/SCID mice. I investigated the influence of MSCs and MAPCs on short term BM homing of freshly isolated $CD34^+$ cells by co-infusion of cells at 1:1 ratio. I also determined if $CD4^+$ and $CD8^+$ cells co-infused at a 1:1 ratio enhanced the homing potential of $CD34^+$ cells to the BM in the NOD/SCID mouse model (Chapter 6).

Chapter 2: General Methods

2.1 GENERAL REAGENTS

All materials, plastics, antibodies, reagents (including composition) and equipment are listed in Appendix I (page 193).

2.2 HSPC cell isolation, culture and characterisation

2.2.1 Primary haemopoietic stem cell source

In accordance with local ethical guidelines, and with local ethics committee approval, peripheral blood stem cells (PBSCs) were collected either from normal subjects donating cells for allografting or from patients being treated for either lymphoma or myeloma on the University College London Hospitals NHS Trust stem cell programme. In the case of normal donors, PBSCs were mobilised with G-CSF. In the case of patients with malignant diseases PBSCs were mobilised with G-CSF and either cyclophosphamide or ESHAP (a chemotherapy regimen containing cisplatin, etoposide, cytarabine and methylprednisolone protocols described in (Velasquez *et al*, 1994;Watts *et al*, 2000)).

2.2.2 $CD34^+$ cell isolation.

$CD34^+$ cells were isolated (Abs recognising the CD34 epitope QBEND/10) using a large scale CliniMACs system described in (Watts *et al*, 2002) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in accordance with manufacturers instructions, by Dr MJ Watts and Mr SJ Ings. A small aliquot of processed cells, surplus to clinical requirements, was then made available for experimental purposes. $CD34^+$ cells were also immunomagnetically selected using the VarioMACS LS column (Miltenyi Biotec). Cells from 1ml of PBSC harvest product were washed once in 10ml of PBS/EDTA solution (Miltenyi Biotec), pelleted by centrifugation (1800rpm for 10 minutes) and then, following aspiration of the supernatant, resuspended in 100µl of anti-CD34 iron/dextran conjugated antibodies (Miltenyi Biotec) and incubated for 20 minutes at 4°C. The VarioMACS LS column, placed in its magnetic retainer, was washed once in 3ml of PBS/EDTA to remove residual azide. The immunolabelled cells were then washed once in 10ml PBS/EDTA and the cell pellet resuspeded in 1ml of PBS/EDTA and loaded onto the column.

Retained $CD34^+$ cells were washed once with 4ml of PBS/EDTA before the column was removed from the magnet. Cells were eluted from the column with a further 1ml of PBS/EDTA which was gently pushed through the column using the plunger provided. Cells were then passed down a fresh column in a similar manner to maximise $CD34^+$ purity. $CD34^+$ cell preparations by both methods, as evaluated by immunofluorescence using anti-CD34-PE (HPCA-2), had an average purity of 93±3%. Cells were cultured either directly or following a period of storage in liquid nitrogen in equal volume of freeze mix (9ml PBS w/o Ca^{++} Mg^{++} and 3ml DMSO chilled for 30 minutes at 4°C followed by the addition of 8ml 20% HSA). Freshly isolated versus cryopreserved cells were compared for CFC output, and *in vivo* homing and engraftment, and no significant difference was found between these 2 sources of cells.

2.2.3 Cell culture.

$CD34^+$ cells were cultured in tissue culture flasks (Nalge Nunc) in X-VIVO 10 (Biowhittaker) and 1% HSA (Immuno Ltd) at a density of $0.5x10^6$/ml supplemented with SCF, Flt3L (100ng/ml) IL-3, IL-6 (20ng/ml) at 37°C and 5% CO_2. In some experiments cells were cultured with SCF, Flt3L (100ng/ml) and TPO (20ng/ml) or SCF, TPO (50ng/ml) and SDF-1α (100ng/ml). In appropriate experiments cells were also cultured on RetroNectin (Takara Shuzo Co., Shiga, Japan) coated 6 well tissue culture plates (Costar). Percentage of $CD34^+$ cells up to 96 hr in culture was 87±4%. The viability of cultured cells was >95% as judged by TB exclusion.

2.2.4 Viability counts

The cell viability of purified $CD34^+$ cell fractions and cultured cells was assessed using a haemocytometer following an appropriate dilution in 0.4% Trypan Blue solution (Sigma).

2.2.5 Cytocentrifuge preparations

Cytocentrifuge preparations were made by adding 0.5 to $1x10^5$ of the cell suspension to 2 drops of 20% human serum albumin in a disposable cytocentrifuge cup (Shandon). The cells were spun onto the slides at 500rpm

for 7 minutes and air dried. Cytospins were stained by standard May-Grunwald-Giemsa for blast cell morphology (Figure 3.1) and visualised with an Olympus inverted microscope.

2.2.6 Cell thaw methods

For some studies cryopreserved $CD34^+$ cells were used. The cryovials were thawed quickly in a 37^0C waterbath and thawed cell suspension transferred to a sterile 20ml conical universal. This was resuspended to 10ml dropwise with X-VIVO 10/4% HSA and pelleted at 1800 for 10 minutes. The cell pellet was resuspended in 1ml X-VIVO 10/4% HSA and cell count, viabilities and CFC performed. $CD34^+$ cells were 'rested' overnight at 4°C in X-VIVO 10/4% HSA at a density of $0.5x10^6$/ml in tissue culture flasks (Nalge Nunc) before use in experiments. HUVECs were thawed in a similar manner except the thawed cell suspension was resuspended to 10ml with Iscoves/20% FCS and pelleted at 1000rpm for 10 minutes.

2.2.7 Clonogenic assays

CFC activity was assessed in semi-solid methylcellulose based media (Methocult H4230 Stem Cell Technologies, Canada) with 20% IMDM and supplemented with GM-CSF 25ng/ml, SCF 10ng/ml and IL-3 30ng/ml. Aliqouts of cells were suspended in 2.5ml of media as indicated and were cultured in 0.5ml aliquots in wells in a 24 well tissue culture plate at 37°C and 5% $C0_2$. Colonies were counted 14 days later for GM-CFC by morphologic criteria (if cell numbers exceeded 64) by means of an inverted stereo microscope (Olympus Optical, London). The appearance of colonies formed are shown in Figure 3.7.

2.2.8 Cell cycle analysis

$CD34^+$ cells were pelleted ($0.5\text{-}1x10^6$/tube) and fixed in 1ml ice-cold 70% methanol in PBS w/o Ca^{++} Mg^{++} overnight at –20°C.The cells were pelleted once more, washed once in PBS w/o Ca^{++} Mg^{++} and then incubated in 0.5ml staining solution for 30 min at 37°C. Samples were then analysed using an Epics-Elite flow cytometer (Beckman-Coulter, UK).

2.2.9 Immunophenotyping

To determine surface expression of CD34, CD38, CXCR4, CD95, VLA4, VLA5, CD31, CD11a, CD3, CD4, CD8, CD45, CD13, SH2, SH4, HLA-DR, HLA-ABC, CD49d and CD44, aliquots of $5x10^4$ cells (except MAPCs due to limited cell numbers $3x10^4$ were stained) were incubated with saturating concentrations (5-20µl) of either specific FITC, PE, RD-1 and PC5 conjugated antibody or the appropriate matching isotype control antibodies in 50µl of PBS w/o Ca^{++} Mg^{++} for 20 minutes at room temperature. Cells were then washed once in 2ml PBS w/o Ca^{++} Mg^{++} and fixed in 200µl of 2% paraformaldehyde. Samples were stored at 4°C in the dark until ready to run on the EPICS Elite flow cytometer (Beckman-Coulter).

2.2.10 Labelling human MPB $CD34^+$ cells with PKH26.

Fresh or cultured MPB $CD34^+$ cells were labelled with PKH26 dye according to manufacturer's (Sigma) instructions with some modifications. Briefly, cells were washed once using 10ml of HBSS without serum and resuspended in 1ml of Diluent C at a concentration of $20\text{-}40x10^6$/ml in a 50ml polypropylene tube. Cells were combined with an equal volume (1ml) of PKH26 dye freshly prepared at 10µM in Diluent C (10µl of PKH26 dye stock at 1mM was added to 1ml of Diluent C in a 15 ml polypropylene tube), and incubated at room temperature for 4 minutes with periodic gentle mixing. Staining was terminated by the addition of an equal volume of FCS for 1 minute, and the labelled cells then washed three times in HBSS/2% FCS. A cell count was performed using trypan blue exclusion on a haemocytometer and the cell recovery after this procedure was between 71-88%. An aliquot of cells before and after PKH26 staining was assayed for CFCs and generated comparable numbers of total colonies (Table 3.6). A small number of cells were also kept from each group to determine the staining efficiency (Figure 3.13). The remaining cells were then injected intravenously into sub-lethally irradiated or non-irradiated NOD/SCID mice (10 million cells per mouse in a maximum of 500µl HBSS).

2.3 Mice and transplantation of human MPB $CD34^+$ cells.

2.3.1 Mice

NOD/LtSz-$PrKdc^{scid}$ (NOD/SCID) mice were housed under positive pressure in a 'ventirack' obtained from Biozone, Kent, UK. 8-10 week old mice were sub-lethally irradiated (325cGy from ^{137}Cs source) and immediately transplanted with the cells resuspended in a maximum volume of 500µl of HBSS by lateral tail vein injection. Mice were sacrificed by CO_2 inhalation at selected intervals post transplantation.

2.3.2 Removal of bones (femurs and tibias) the spleen, lungs and livers from NOD/SCID mice

Once the mouse had been sacrificed by CO_2 inhalation it was positioned on its back and 70% IMS used to wet fur thoroughly. This step decreased the possibility of contaminating cell preparations with fur. A slit was cut in the fur just below the rib cage without cutting the peritoneal membrane. Skin was firmly grasped and peeled back to expose hind limbs. Using sterile sharp dissecting scissors, the knee joint was cut in the center. The ligaments and excess tissue were cut. The femur was grasped with forceps and femur cut near hip joint. The ends of the long bones were trimmed to expose the interior marrow shaft. We obtained a clean bone and one tibia was placed into 1ml fomalsaline. The other long bones (2 femora and 1 tibia), were placed in 1ml of 10% FCS/RPMI supplemented with 20U/ml heparin. For the removal of the spleen, the skin was peeled back to expose the peritoneum as above. Using a sterile dissecting scissors a incision was made in the peritoneal membrane. The spleen was gently pulled out and detached by cutting the splenic blood vessel. The lungs and livers were also removed and placed in formalsaline and 10% FCS/RPMI supplemented with 20U/ml heparin, as above.

2.3.3 Analysis of murine tissues for human progenitor cell homing.

BM cell suspensions were prepared from two femurs and one tibia and from spleens, lungs and livers. BM was flushed out using HBSS and a 5ml syringe (Terumo) attached to a 26 gauge needle (BD) and put through a 40µM cell

strainer (BD) and collected into a 50ml polypropylene tube containing 0.5ml of FCS. The spleen, lungs and livers were cut up finely and eased through a 40μM cell strainer using a plunger of a sterile 1ml syringe (Terumo) and collected into a 50ml polypropylene tube containing 0.5ml FCS. The cell suspensions were pelleted at 1600rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended and incubated on ice for 5 minutes. RBC were lysed in 2ml of red cell lysis buffer (155mM NH_4Cl, 20mM $NaHCO_3$, 1mM EDTA ;BDH, Poole, UK) for 4 minutes on ice and rescued with 10ml of HBSS supplemented with 1% Glucose and 1% BSA (RBC lysis rescue buffer). The cells were pelleted at 1600rpm for 10 minutes and washed once in 10ml of HBSS /5% FCS. Cell counts and viability were performed (see section 2.2.4) and set up in clonogenic assays in methylcellulose (Methocult (H4230; Stem Cell Technologies Inc., Vancouver, Canada), with 20% IMDM)) supplemented with GM-CSF 25ng/ml, SCF 10ng/ml and IL-3 30ng/ml. These conditions are selective for human colonies as no colony growth was seen using BM from control saline-injected animals. Purified $CD34^+$ cells ($5.0x10^2$/ml) or harvested BMMNCs ($2\text{-}4x10^5$/ml) were plated in quadruplicates. Total human GM-CFC colonies per animal were calculated based on the assumption that 1 tibia and 2 femurs represent 12% of total BM (Chervenick *et al*, 1968) and were expressed as a percentage of the number of GM-CFC infused (see calculation below). To quantify the level of homing in other tissues, GM-CFC were counted and expressed per 10^6 cells. This is because parts of the spleens, lungs and liver were removed for immuno-histochemistry.

Calculation for % homing of GM-CFC to BM

$$\% \text{ of infused in BM} = \frac{[(A/B)*C]*D}{E} X100$$

A= Number of GM-CFCs
B= Number of BMMNCs seeded
C= Total murine BMMNC count
D= 8.333 (2 femurs & 1 tibia represent 12% of total BM, therefore 100/12=8.33)
E= Total number of GM-CFCs infused

2.3.4 Analysis of human cell engraftment.

Six weeks post infusion, mice were sacrificed and single cell suspensions prepared from the BM of two femurs and one tibia (see section 2.3.3). One million BMMNCs were aliquoted into capped plastic test tubes for flow cytometry and were incubated with 100µl of mouse serum in PBS w/o Ca^{++} Mg^{++}/1%BSA for 15 min on ice. The cells were washed once with PBS w/o Ca^{++} Mg^{++}/1% BSA and stained at 4^0C in the dark with anti-human CD45-PerCP, CD34-FITC, CD38-FITC, CD19-PE, CD13-FITC, CD3-FITC, CD2-PE in appropriate combinations (Table 2.1) in 100µl staining buffer (PBS w/o Ca^{++} Mg^{++} / 1% BSA / 0.1% sodium azide). A sample from each mouse was also stained with isotype-matched control antibodies (Table 2.1). Cells were washed twice with PBS w/o Ca^{++} Mg^{++}/1% BSA and fixed in 200-400µl of 1% paraformaldehyde. Samples were stored at 4°C in the dark until flow cytometry. Samples were analysed on the Epics-Elite flow cytometer (Beckman-Coulter, High Wycombe, UK), and at least 50,000 events were acquired and analysed using Elite Workstation Analysis Software Version 4.5. An animal was considered to have successfully engrafted if the BM contained >1% $CD45^+$ cells.

Table 2.1. Antibody panel combinations for engraftment analysis

Tube 1	Isotype controls	volume per test tube
	IgG_1 FITC	5µl
	IgG_1 PE	5µl
	IgG_1 PerCP	5µl
	Staining buffer	85µl
Tube 2	CD19/13/45	
	CD19 PE	20µl
	CD13 FITC	10µl
	CD45 PerCP	5µl
	Staining buffer	65µl
Tube 3	CD34/38/45	
	CD34 FITC	10µl
	CD38 PE	5µl
	CD45 PerCP	5µl
	Staining buffer	80µl
Tube 4	CD3/2/45	
	CD3 FITC	5µl
	CD2 PE	5µl
	CD45 PerCP	5µl
	Staining buffer	85µl

2.3.5 Immunohistochemistry

Tissues (BM, Spleen and Lungs) from transplanted animals and controls (saline) were fixed in 1ml of 10% formalsaline. For histopathological examination, mouse tibias were decalcified in 10% formic acid while stirring overnight and then all tissues were embedded in paraffin and sectioned. Slides were stained with human anti-CD45 RB (Dako). For CD45-RB immunohistochemistry, formalin-fixed paraffin sections were dewaxed

(xylene and 100% alcohol for 2 minutes x2) and treated with peroxidase block for 10 minutes at room temperature (12ml methanol + 200µl H_2O_2 at 30%). Slides were washed thoroughly in tap water and heat retrieval of antigen was done using a domestic pressure cooker in 3L citrate buffer at pH6 for 2 minutes. MOM Immunodetection Kit (PK-2200 Vector Laboratories, Burlingame, CA, USA) was used to block background as per manufacturers instructions. Briefly, sections were incubated with MOM mouse Ig blocking reagent for 1 hour and washed three times with Tris Buffered Saline (TBS). Sections were then incubated with MOM Diluent for 5 minutes and excess MOM Diluent was tipped off. Sections were incubated with mouse anti-human CD45-RB (Dako M0833) for 30 minutes at 1/100 and washed twice with TBS, then incubated with a MOM biotinylated anti-mouse IgG for 10 minutes (Vector Labs), washed with TBS and finally incubated with Vectastain Avidin Biotin Complex for 5 minutes (ABC; Vector Labs). Sections were washed twice with TBS and developed with 3',3'-diaminobenzidine (DAB; Ken-En-Tec A/S, Denmark) substrate (1 DAB tablet was dissolved in 10ml distilled water in the fridge for 5 minutes and 10µl of H_2O_2 added just before use). Colour reaction was stopped by washing slides in tap water. Sections were lightly counterstained with haematoxylin for 4 minutes and differentiated in 1% acid alcohol for 1-2 seconds. The sections were then put through series of alcohol steps (70, 95, 100% and xylene) for 1 minute. Slides were mounted with Styrolite mounting medium (BDH) and analysed under 400x magnification.

2.4 Statistical analysis.

Data are expressed as median and range of multiple measurements or mean ± SEM. The statistical significance between groups was carried out using the nonparametric Mann-Whitney U Test (on GB STAT for Apple Macintosh, version 6.5.4, Dynamic Microsystems, Inc) and unpaired 2-tailed Student's t-test (on Excel for PC) and a P value of <0.05 was considered significant.

2.4.1 Statistical test selection

The t-test (e.g. students t-test), like many statistical tests, assumes that you have sampled data from populations that follow a Gaussian bell shaped distribution. An alternative approach does not assume that data follow a Gaussian distribution. In this approach, values are ranked from low to high and the analyses are based on the distribution of ranks. These tests, called non-parametric tests, are appealing because they make fewer assumptions about the distribution of the data. The Mann-Whitney U test, also called the rank sum test, is a non-parametric test that compares two unpaired groups. If the data are paired or matched, then you should choose a Wilcoxon matched paired test instead (Table 2.2). The Mann Whitney U test compares the medians of two groups .

Table 2.2 - Statistical test selection

Test	Paired	Non-parametric	Unequal Variance
Unpaired t-test	No	No	No
Paired t-test	Yes	No	N/A
Mann-Whitney U test	No	Yes	N/A
Wilcoxon test	Yes	Yes	N/A

Chapter 3: Effect of cytokine exposure on the engraftment and homing of $CD34^+$ cells in the NOD/SCID mouse model

3.1 INTRODUCTION

Cytokine exposure alters engraftment potential of HSC

In recent years, much effort in stem cell research has focussed on *ex vivo* manipulation of haemopoietic stem/progenitor cells (HSPCs). The potential advantages for *ex vivo* HSPC expansion are many and include the following: (i) decreased time to haemopoietic recovery after chemotherapy or transplantation; (ii) an increased long term repopulating cell (LTRC) component of small grafts, such as umbilical cord blood (UCB) grafts, or grafts from patients heavily pretreated with chemotherapy; (iii) the removal of cancer cells from a graft; and (iv) the genetic modification of LTRCs either to replace defective genes, or to express therapeutic genes of interest (reviewed in (Verfaillie, 2002a)). $CD34^+$ cells from granulocyte-colony stimulating factor (G-CSF) mobilised peripheral blood (MPB) grafts have become the preferred source of stem cells for transplantation because of earlier neutrophil and platelet recovery, thought to be due to the increased number of short term repopulating cells (STRCs) (Korbling & Anderlini, 2001), and hence represent an attractive model for *ex vivo* expansion.

Cell expansion requires exposure to cytokines as this is required for maintenance of primitive haemopoietic progenitors viability *ex vivo*. In addition, cell expansion and gene transduction strategies require cells to pass through mitosis (Lewis & Emerman, 1994), which also involves cytokine stimulation. Cytokine activation may, however have detrimental effects on subsequent engraftment (Liu *et al*, 2003;Young *et al*, 2001). Exposure of stem cells to growth factors *in vitro* reduces the *in vivo* repopulating ability of these cells, as demonstrated in murine and xenogeneic systems (van der Loo & Ploemacher, 1995;Peters *et al*, 1996;Szilvassy *et al*, 1999;Szilvassy *et al*, 2000;Guenechea *et al*, 1999), and as evidenced in studies on retrovirally transduced cells (Dorrell *et al*, 2000;Demaison *et al*, 2000). In the xenogeneic model (Guenechea *et al*, 1999), fresh UCB $CD34^+$ cells were compared with cells stimulated for 6 days with IL-3, IL-6 and SCF or IL-11, SCF and Flt3L. A significant impairment in the short term repopulation (20 days post transplantation) of NOD/SCID animals was associated with the

transplantation of *ex vivo* expanded cells when compared with the fresh cells. Such observations have raised serious concerns regarding the use of cultured HSPC products for clinical transplantation.

In vivo homing of HSC

Much work, therefore has focussed on discovering the basis for the reduced engraftment of cytokine exposed cells. A frequently made assumption is that the reduced engraftment of cytokine exposed HSPC results from changes in their homing ability. It has been hypothesised that the failure of cultured transplanted haemopoietic cells to efficiently engraft *in vivo* may be related to a change in the ability of such cells to negotiate the BM endothelial cell barrier (i.e. HSCs acquire a homing defect). Some support for this comes from murine studies where seeding of HPC/CFC to the BM/spleen was found to decrease following *ex vivo* cytokine exposure (Szilvassy *et al*, 1999;van der Loo & Ploemacher, 1995). In one study (van der Loo & Ploemacher, 1995), the effect of cytokine exposure on the short term seeding efficiency of murine haemopoietic stem cells to BM and spleen was assessed using the CAFC assay. Preincubation with cytokines for 2-3 hr at 37°C led to a substantial decrease in seeding efficiency compared with control cells kept on ice. Such changes in seeding efficiency may be related to the finding that *ex vivo* culture induces a down regulation of β1 integrin on mouse haemopoietic cells (Szilvassy *et al*, 2001b).

Other workers have reported that *ex vivo* culture of progenitors leads to changes in expression of members of the β1 integrin family as well as the chemokine receptor CXCR4, which play key roles in homing and engraftment of HSPC (Giet *et al*, 2001;Glimm *et al*, 2000;Orschell-Traycoff *et al*, 2000;Peled *et al*, 2000;Papayannopoulou & Nakamoto, 1993;Papayannopoulou *et al*, 1998;Levesque *et al*, 1995). Changes in the expression of such critical surface receptors in response to cytokine stimulation could alter stem/progenitor cell homing properties and reduce engraftment by several mechanisms including; (i) random egress of transplanted cells into non haemopoietic organs or (ii) inability of transplanted

cells to localise or secure anchorage in bone marrow (Szilvassy *et al*, 1999;Papayannopoulou & Craddock, 1997). There has not yet been a systematic study of the effect of cytokine stimulation on the homing abilities of adult human HSPC. Most studies have been carried out using UCB (Liu *et al*, 2003;Kerre *et al*, 2001) or murine stem cells (Hendrikx *et al*, 1996;Cui *et al*, 1999;Szilvassy *et al*, 2001a;Lanzkron *et al*, 1999;Cerny *et al*, 2002;Dooner *et al*, 2004). However, there is evidence that the primitive progenitors in mobilised PB are qualitatively different from those found in bone marrow (BM) (van der Loo *et al*, 1998a;Verfaillie *et al*, 2000) and UCB (Yong *et al*, 1999;Holyoake *et al*, 1999;Zheng *et al*, 2003).

In vitro transmigration of HSCs

Previous studies in this area using an *in vitro* assay have focussed on the early critical step of transendothelial migration, whereby HSPC egress from the circulation into haemopoietic tissue (Yong *et al*, 1998). They have shown that while $CD34^+$ cells adhere readily to the endothelial surface, they do not undergo transmigration unless activated by cytokines that induce cell division (Yong *et al*, 1998). The difficulty of assessing homing using an *in vitro* assay arises because it cannot totally mimic the complex process of homing which involves circulation through blood, recognition and extravasation through BM vascular endothelium, and migration into a supportive microevironment. Hence many investigators have directed their efforts to the development of small animal models of human HSPC transplantation (see section 1.3.2.3).

Homing studies

In general two types of homing studies have been carried out. The first is one in which whole unseparated marrow is infused and a surrogate assay (see section 1.3.2.1) is used as marker for stem cells (Frenette *et al*, 1998;Szilvassy *et al*, 1999;Oostendorp *et al*, 1999). In the second, purified stem cells are labelled (for e.g. with fluorescent aliphatic dye PKH26) to enable them to be tracked after infusion (Hendrikx *et al*, 1996;Lanzkron *et al*, 1999;Cui *et al*, 1999;Szilvassy *et al*, 2001b). The first approach raises concerns for the validity of the surrogate assay and the possibility that the biological phenotype of the cell may change after engraftment. The second

approach is difficult because of the relatively large number of purified stem cells needed.

Chapter Aims

To effectively use haemopoietic stem cells and progenitor cells for clinical gene transfer (Williams & Smith, 2000), and for graft expansion (Lewis *et al*, 2001), it is important to understand whether and how homing and engraftment of haemopoietic cells is affected by *ex vivo* manipulation. The initial aims of the project were to characterise the changes in engraftment behaviour in conjunction with alteration in adhesion molecule expression and homing, of MPB $CD34^+$ cells after culture with SCF, Flt3L, IL-3 and IL-6. This cytokine combination was selected because it has been optimised for support and maintenance of primitive HSCs and used for retroviral transductions (Conneally *et al*, 1997;Dao *et al*, 1997;Demaison *et al*, 2000). We quantified short term homing of human CFC to different organs and of PKH26 labelled $CD34^+$ cells homing to the BM in NOD/SCID animals in parallel with engraftment studies carried out on the same cells. We have investigated whether the reduced engraftment of cytokine stimulated $CD34^+$ cells is directly related to an alteration in short term organ specific homing.

3.2 RESULTS

3.2.1 Effect of cytokine activation on $CD34^+$ cell expansion and morphology

$CD34^+$ cells were cultured in X-VIVO 10 (a serum free medium suitable for cultivation of stem cells) 1% HSA and supplemented with the following growth factors, SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml) and IL-6 (20ng/ml), referred as the 4 cytokine mixture here after. $CD34^+$ cells cultured in this medium proliferated rapidly, as shown in Table 3.1. At 48 hr of culture cells had not expanded significantly (1.1 ± 0.3 fold expansion, mean $\pm$ sem) but by day 3, cells had expanded 4-fold. During this culture period, the morphological features of $CD34^+$ cells were observed by staining cytospin preparations with MGG, as shown in Figure 3.1. Freshly isolated $CD34^+$ cells were primitive in appearance with a scanty cytoplasm and high nuclear to cytoplasmic ratio. By 24hr, cells had begun to go into cycle as demonstrated by the presence of the odd mitotic cell. At 48hr of culture cells were larger

with cytoplasmic basophilia and the presence of primary azurophilic granules and vacuoles.

Table 3.1 - Culture and fold expansion of human MPB $CD34^+$ cells.

Time of culture (hr)	Fold expansion (mean ± SEM)
48	1.1 ± 0.3
72	4 ± 0.8

$CD34^+$ selected PBSC progenitors were cultured in X-VIVO 10/1% HSA supplemented with SCF and Flt3L 100ng/ml, IL-3 and IL-6 20ng/ml at a cell density of $0.5x10^6$/ml. Fold expansion of input numbers is shown for 5 separate experiments.

3.2.2 Effect of cytokine expansion on CFC numbers.

I assessed the functional activity of freshly isolated haemopoietic progenitors and cells cultured for 1, 4, 24 and 48hr in the 4 cytokine mix by methylcellulose colony assay *in vitro*. As shown in Table 3.2, up to 48hr of culture, cells generated comparable numbers of total colonies as fresh cells, n=2, however, culture for longer period (72 and 96 hours) also resulted in a comparable frequency of CFC (Table 3.3).

Table 3.2 - Effect of culture duration on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
Time in culture (hr)	Expt 1	Expt 2
0	69	183
1	54	N/D
4	75	182
24	81	183
48	97	142

Freshly isolated $CD34^+$ cells (0) were cultured for 1, 4, 24 or 48 hr in X-VIVO 10/1% HSA supplemented with the 4 cytokine mix as described in Chapter 2 General Methods. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

Table 3.3 - Effect of culture time (3 or 4 days) on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	27	127
72hr cultured	109	123

	No. of GM-CFC per 1000 cells		
	Expt 1	Expt 2	Expt 3
Fresh	117	60	53
96hr cultured	131	70	75

Freshly isolated $CD34^+$ cells were stimulated with the 4 cytokine mix for 72 and 96 hrs. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for independent experiments.

3.2.3 Human MPB $CD34^+$ cells placed in liquid culture undergo changes in expression of surface receptors

Concomitant with the morphological changes observed in liquid culture (Figure 3.1), changes in expression of cell surface proteins occurred during cytokine stimulation. Freshly isolated $CD34^+$ cells were compared to cells stimulated for 48hr with the 4 cytokine mixture. There was little change in surface expression of the early haemopoietic progenitor marker CD34 and the differentiation marker CD38 (Figure 3.2). In contrast, surface expression of the β1 integrins VLA-4 and VLA-5 were upregulated upon cytokine exposure. VLA-4 expression on $CD34^+$ cells increased from 23 ± 9 to 93 ± 2% ($P<0.01$) after culture, while VLA-5 increased from 20 to 88% (n=2) for fresh and cytokine cultured cells, respectively. In contrast there was no significant change in the expression of CD11a (6 to 20%) or of PECAM-1, also known as CD31 (59 ± 20 to 87± 4%) after 2 days of culture (Figure 3.2). Other surface receptors which were altered by cytokine exposure of $CD34^+$ cells are Fas (also known as CD95) and CXCR4 (7 span transmembrane receptor). Fas expression on $CD34^+$ cells increased from 6±1 to 65±9% after 2 days of culture, ($P<0.01$, n=3) and CXCR4 expression on $CD34^+$ cells also increased from 3 ± 1 to 51 ± 15% after 2 days of culture, $P<0.05$.

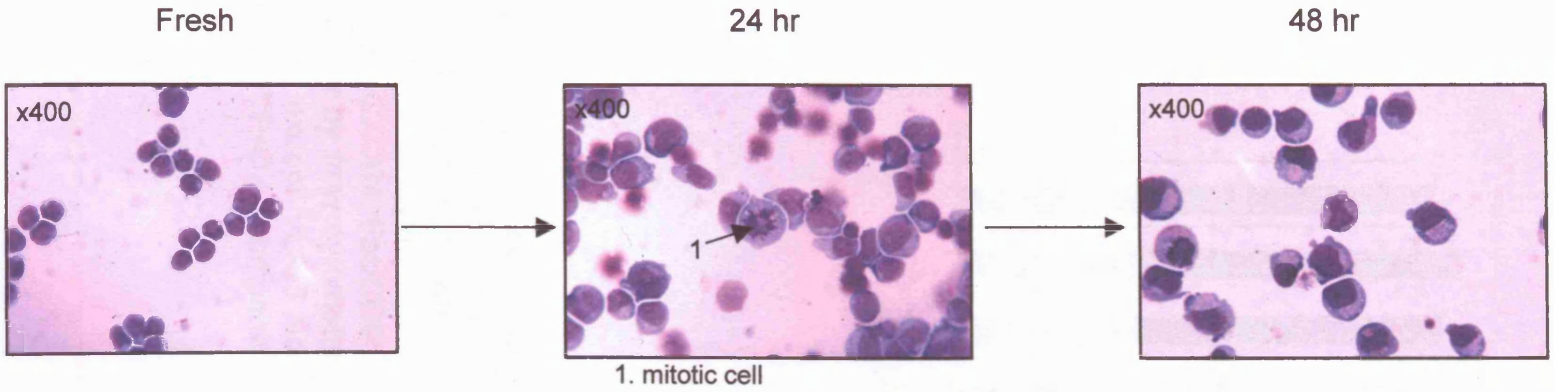


Figure 3.1 - Morphology of selected MPB CD34⁺ cells and after short term suspension culture. CD34⁺ selected PBSCs were cultured in X-VIVO 10/1% HSA supplemented with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) at $0.5x10^6$/ml for 24 and 48 hr. Cells were spun onto glass slides and stained with MGG.Representative cytospins of fresh and cultured cells are shown, made on the indicated hours. Cells were viewed with the magnification as indicated in each plate.

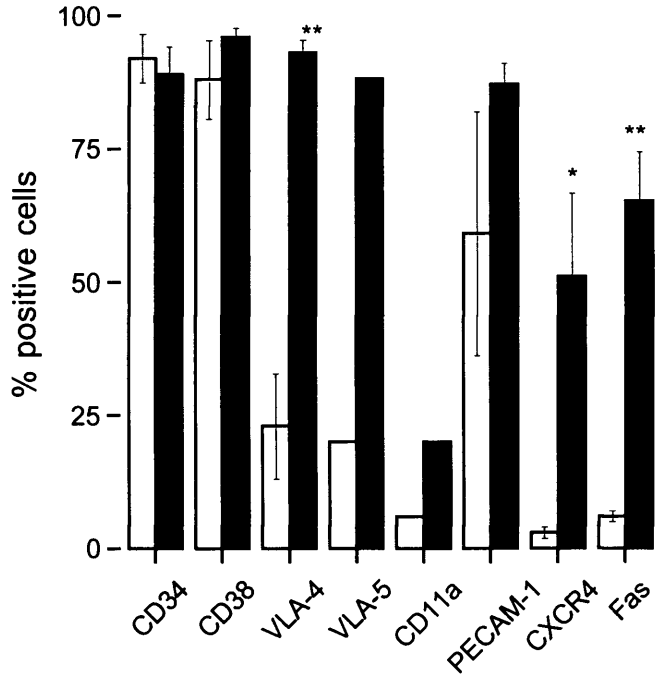
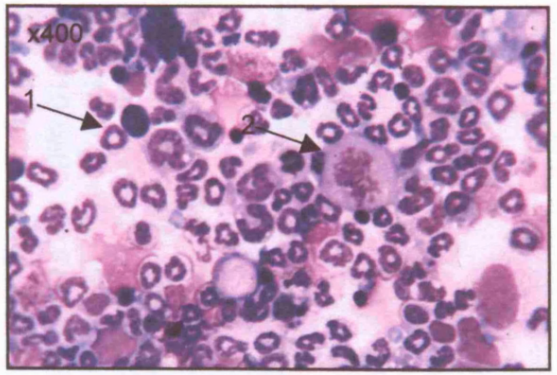


Figure 3.2 - Effect of cytokine exposure on the expression of surface receptors in MPB CD34$^+$ cells. Freshly isolated CD34$^+$ cells (open bars) or cultured cells (48hr stimulation with SCF, Flt3L 100ng/ml and IL-3, IL-6 20ng/ml, solid bars) were stained with FITC or PE-conjugated anti-human CD34, CD38, CXCR4, Fas/CD95, VLA-4, VLA-5, CD11a and PECAM-1 mAb and analysed by flow cytometry. Each bar represents mean ± SEM of 3 experiments, except VLA-5 and CD11a mean of 2 experiments shown (*P<0.05 and **P<0.01 compared to fresh cells, Student t-test)

3.2.4 Cytokine activation reduces engraftment of MPB CD34$^+$ cells in NOD/SCID animals

In order to confirm that cytokine stimulation of human MPB CD34$^+$ cells compromises engraftment potential, I studied the engraftment of freshly isolated and cytokine activated MPB CD34$^+$ cells in NOD/SCID animals by FACS analysis of murine BM (Figure 3.3) performed at 6 weeks post transplantation. Freshly isolated CD34$^+$ cells were stimulated with the 4 cytokine mixture (Table 3.4b) for 2-5 days (Table 3.4a) and tested for their engraftment potential, in comparison with fresh cells. As shown in Table 3.4a and Table 3.1, cytokine exposure (for >48 hrs) led to an increase in cell number (130% to 520% of initial) hence in order to avoid any dilutional effect on stem cells initially present in the graft, the numbers of cytokine exposed CD34$^+$ cells infused represented the expanded equivalent of fresh cells. Table 3.4a summarises the results of the first set of experiments performed in this way. Animals which received cytokine activated cells showed lower engraftment (median 0.7%, range 0.0-22.7% CD45$^+$ cells in murine BM) with only 3 out of 7 animals engrafting, while all animals (7 of 7) receiving fresh cells engrafted (median 4.6%, range 2.0-78.4% CD45$^+$ cells in murine BM), n=2, $P<0.05$. I also performed a second series of experiments in which equal numbers of fresh or cultured cells were infused (Table 3.4b). Although the engraftment levels were generally lower (median of 1.7% [range 1.2-16%] and 0.1% [range 0-0.5%] for fresh and cultured cells, respectively), cultured cells again demonstrated a marked engraftment defect, such that no animal (0 of 5 animals) receiving cultured cells engrafted n=2, $P<0.01$. Assessment of engraftment by FACS analysis was confirmed by colony assays on cells recovered from murine BM (Figure 3.3) using human-specific cytokines (GM-CSF, SCF and IL-3). As shown in Figure 3.4, BM from mice transplanted with fresh CD34$^+$ cells formed a median of 31 GM-CFC colonies per 10^5 murine BM cells plated, in comparison to colony yield from BM of animals transplanted with cultured cells which formed only a median of 2 colonies per 10^5 cells, (n=2, $P<0.01$).



1. Neutrophil
2. Megakaryocyte

Figure 3.3 - NOD/SCID mouse bone marrow single cell suspension. Bones removed from NOD/SCID mice transplanted with cells were flushed with HBSS and the cells recovered were lysed with RBC lysis buffer. Cells were spun onto glass slides and stained with MGG. Representative cytospin of murine BM cells is shown. Cells were viewed under the magnification indicated in the plate.

Table 3.4 - Effect of cytokine stimulation on the engraftment of $CD34^+$ cells in NOD/SCID mice.

(a)

Expt. No.	Cells/mouse (10^6) Fresh	Cells/mouse (10^6) Post culture	% $CD45^+$ cells in mouse BM Fresh	% $CD45^+$ cells in mouse BM Post culture
1	0.5	0.7	2.0, 2.3	0.0, 1.3
2	0.5	2.6	4.6	2.7
1	1.0	1.3	2.1, 10.5	0.1, 0.1
2	1.0	5.2	72.3, 78.4	22.7, 0.7
Median (range)			4.6 (2.0-78.4)	0.7 (0.0-22.7)*
Animals engrafted			7 of 7	3 of 7

(b)

Expt. No.	Cells/mouse (10^6)	% $CD45^+$ cells in mouse BM Fresh	% $CD45^+$ cells in mouse BM Post culture
1	0.5	1.4, 1.2	0.1
1	1.0	5.8, 16.2	0.1, 0.0
2	1.0	1.9, 1.3	0.5, 0.1
Median (range)		1.7 (1.2-16.0)	0.1 (0.0-0.5)**
Animals engrafted		6 of 6	0 of 5

Freshly isolated $CD34^+$ cells (0.5 or 1.0 x 10^6/animal) or the expanded equivalent number (a) or equal number (b) of cultured cells were infused into sub-lethally irradiated NOD/SCID mice. Cultured cells were stimulated with SCF (100ng/ml), Flt3L (100 ng/ml), IL-3 (20ng/ml) and IL-6 20 ng/ml) in X-VIVO 10 with 1% FCS for 2-5 days (a) or 48 hours (b). For all experiments engraftment was assessed at 6 weeks post transplantation by immunophenotype analysis of BM cells as detailed in Chapter 2 General Methods. An animal was considered to have successfully engrafted if $CD45^+$ cells comprised $\geq$ 1% of BM cells. Data are given for each animal, and also as median and ranges of each group as indicated. *$P<0.05$ and **$P<0.01$ compared to fresh cells, Mann-Whitney U Test.

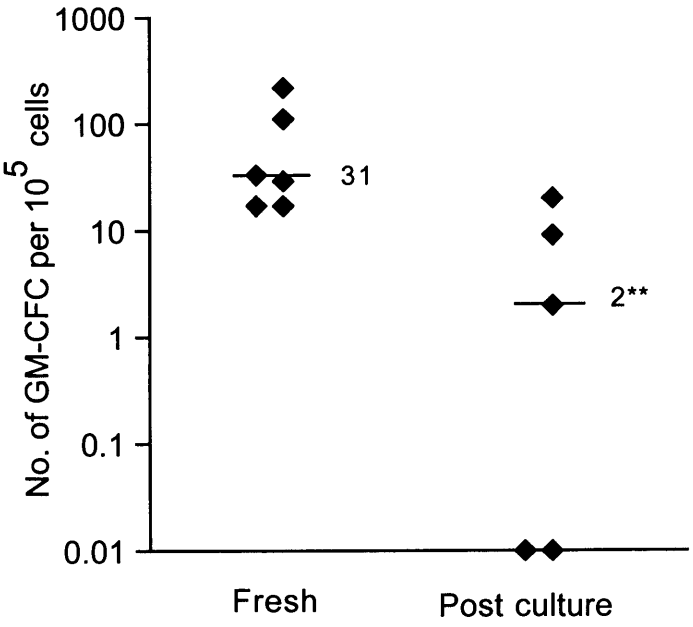


Figure 3.4 - Effect of cytokine stimulation on the engraftment of $CD34^+$ cells in NOD/SCID mice. Engraftment was assessed at 6 weeks post transplantation in mice that received freshly isolated $CD34^+$ cells or cultured cells (48hr stimulation with SCF, Flt3L (100ng/ml) and IL-3, IL-6 (20ng/ml)) 0.5 or 1.0 x 10^6/animal. Bone marrow cells were assayed for human colony formation (as detailed in Chapter 2 General Methods). Data are given for each animal in 2 independent experiments and the median for each group is indicated. **P<0.01 compared to fresh cells, Mann-Whitney U Test.

3.2.5 Multi-lineage engraftment potential of MPB CD34$^+$ cells in the NOD/SCID mice

To compare the *in vivo* differentiation potential of engrafting fresh and post cultured CD34$^+$ cells (Table 3.4a&b), the phenotypic profile of CD45$^+$ cells in BM of recipient mice was determined by 3 colour immunostaining. As shown in Figure 3.5 and 3.6, multi-lineage engraftment was demonstrated in animals which engrafted with either fresh or cytokine activated cells, although the skewing to B-lineage engraftment seen using fresh CD34$^+$ cells was not evident in animals transplanted with cytokine activated cells (Figure 3.5). Multilineage engraftment is shown for both expanded equivalent number (Figure 3.5) and equal number (Figure 3.6) of cultured cells. Expression of CD19 and CD13 (11% and 30%, respectively, for fresh CD34$^+$ and 6% and 12.4% for cultured CD34$^+$ cells recipients) demonstrated the presence of engrafted cells in lymphoid and myeloid lineages, respectively. Expression of CD2 and CD3 (15% and 2%, respectively, for fresh CD34$^+$ and 10% and 9% for cultured CD34$^+$ cells recipients) demonstrated the presence of engrafted cells with T cell potential (Figure 3.6).

3.2.6 Cytokine activation impairs short term homing of CD34$^+$ cells to BM in NOD/SCID animals

Initial experiments were done to assess the localisation of freshly isolated CD34$^+$ cells in the BM, spleen and lungs at 1 and 24 hours post-transplantation. Mononuclear cells recovered from murine BM, spleen and lungs 24hr post transplantation were plated into semisolid culture for enumeration of human CFC, as described in materials and methods. Aliquots of pre-infusion CD34$^+$ cells were set up in parallel methylcellulose culture and used as reference in order to quantify homing efficiency of CFC. The morphology and number of colonies was determined 14 days later. Examples of the typical appearance of GM-CFCs are shown in Figure 3.7. GM colonies formed by purified CD34$^+$ cells and by CD34$^+$ cells recovered from the BM and spleen were similar in appearance, while CD34$^+$ cells recovered from the lungs formed colonies which were less compact and smaller in size.

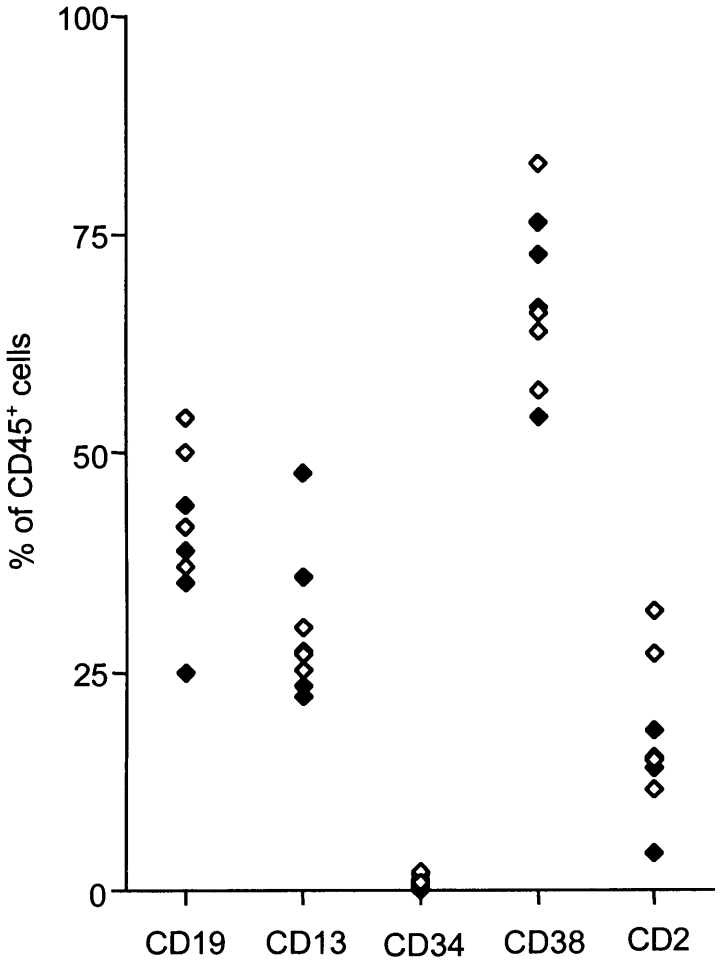


Figure 3.5 - Effect of cytokine stimulation on the multi-lineage engraftment of expanded equivalent number of cultured CD34$^+$ cells in NOD/SCID mice. Lineage specific engraftment of fresh (open symbols) and cultured cells (stimulated with SCF, Flt3L (100ng/ml) and IL-3, IL-6 (20ng/ml) for 2-5 days, closed symbols) was assessed by flow cytometry. The percent of huCD45$^+$ cells (Table 3.2a) that also expressed human lymphoid, myeloid and progenitor surface markers is shown. Data are given for each animal from two independent experiments.

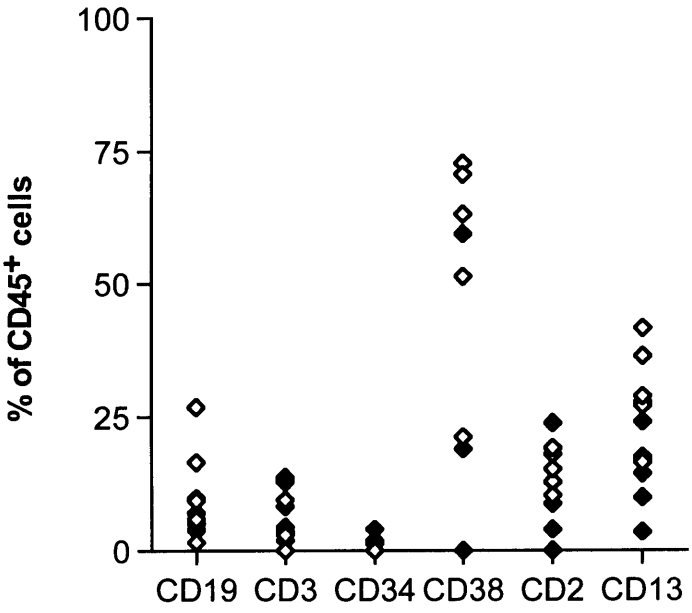


Figure 3.6 - Effect of cytokine stimulation on the multi-lineage engraftment of equal number of cultured CD34$^+$ cells in NOD/SCID mice. Lineage specific engraftment of fresh (open symbols) and cultured cells (48hr stimulation with SCF, Flt3L (100ng/ml) and IL-3, IL-6 (20ng/ml), closed symbols) was assessed by flow cytometry. The percent of huCD45$^+$ cells (Table 3.2b) that also expressed human lymphoid, myeloid and progenitor surface markers is shown. Data are given for each animal from two independent experiments.

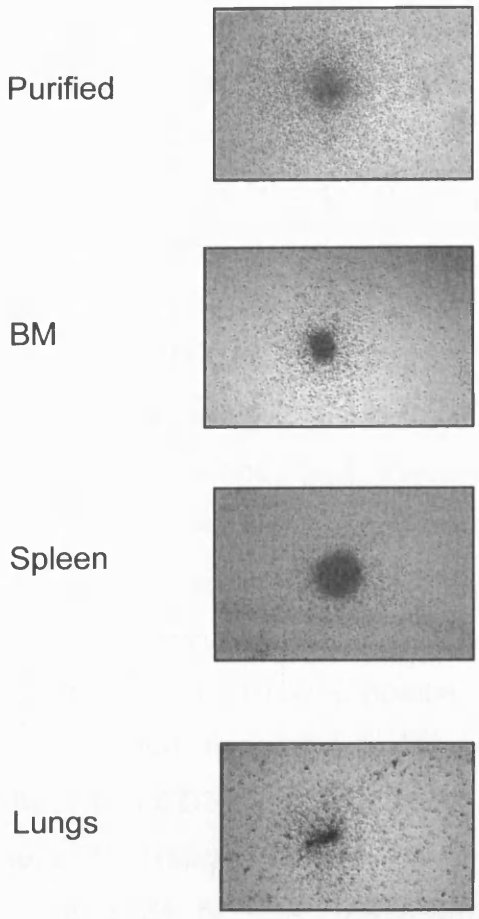


Figure 3.7 - Appearance of human CFC in methylcelluose colony assay. Methylcellulose cultures were viewed at 14 days using an inverted stereo microscope (Zeiss). Colonies from a representative experiment. White cell colonies were scored as GM-CFC if cell numbers exceeded 64. High power view of GM-CFC colonies from purified $CD34^+$ cells and from NOD/SCID mice BM, spleen and lungs 24 hr post transplantation. Magnification x100

As seen in Figure 3.8, homing of CFC to BM (Figure 3.8a) and spleen (Figure 3.8b) was evident at 1 hr post infusion but the numbers of CFC increased substantially at 24 hrs. In contrast, while CFC accumulated in the lungs at 1 hr, numbers were reduced at 24 hrs (Figure 3.8c), suggesting that this represented transient accumulation rather than active homing. To investigate the effect of cytokine exposure, $CD34^+$ cells were incubated with the 4 cytokine mixture for 2-5 days and the expanded equivalent (2, or 5 x 10^6) of $CD34^+$ cells was infused per animal. Cytokine stimulation resulted in a marked reduction in the homing of CFC to the BM when assessed at both 1 and 24 hrs (fresh GM-CFC median 2.0% (range 0.5-2.4) and 2.8% (1.9-6.1) at 1 and 24hr respectively vs 0.1% (0.0-0.2) and 0.3% (0.0-0.7) for cultured cells, n=3, $P<0.01$ for both time points, Figure 3.8)). In contrast, cytokine stimulated CFC showed significantly increased localisation to lung tissue, at both 1 ($P<0.05$) and 24hr post infusion ($P<0.01$). Homing of fresh and post cultured cells to the spleen was also analysed, as the spleen is a haemopoietic site in the mice. Cytokine activated $CD34^+$ cells also showed significant reduction in homing to the spleen (Figure 3.8B). As shown in Figure 3.8B, fresh $CD34^+$ transplanted mice formed a median of 111 (range 15-160) and 205 (range 75-415) GM-CFC colonies per 10^6 murine spleen cells at 1 and 24 hr post transplantation, whereas cultured $CD34^+$ transplanted mice formed significantly less colonies with a median of 15 (range 10-48) and 33.5 (range 8-135), for 1 and 24 hr post transplantation, respectively. The BM homing defect was evident after 2 days and remained unaltered up to 5 days of cytokine stimulation (Figure 3.9). A look at a detailed time course of cytokine exposure (1, 4, 24 and 48hr) showed that homing of CFC to the BM was significantly impaired by 24 hours following cytokine stimulation (mean of 0.6% of infused GM-CFC in BM), and had already begun to fall at 4 hours (Figure 3.10). The reduction of CFC homing to the spleen followed a similar time course of cytokine exposure. As shown in Figure 3.11, fresh $CD34^+$ transplanted mice formed a mean of 65 GM-CFC colonies per 10^6 murine spleen cells, whereas 24 and 48hr cultured $CD34^+$ transplanted mice formed significantly lower number of colonies with a mean of 8 and 6, respectively.

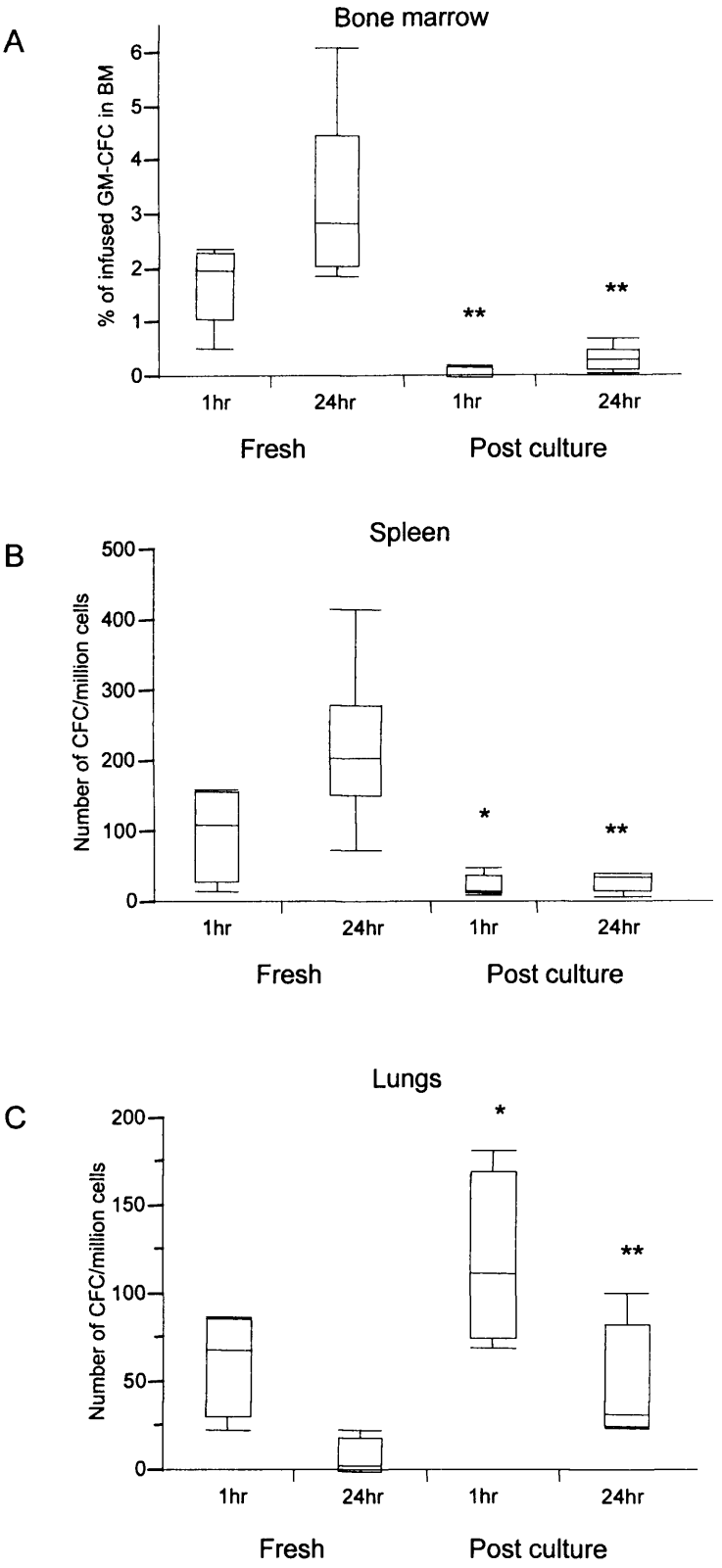


Figure 3.8 - Homing of freshly isolated and cytokine stimulated CD34⁺ cells. Cell suspensions from the BM, spleen and lungs of transplanted NOD/SCID mice recovered 1hr and 24hr after transplantation were assayed for colony formation using human specific cytokines. Number of CFC in lungs and spleen were corrected for the cell expansion after cytokine culture. Median and interquartile range of 4-7 animals pooled from 3 experiments are shown. (*P<0.05, **P<0.01, ***P<0.001 compared to fresh cells, Mann-Whitney U Test)

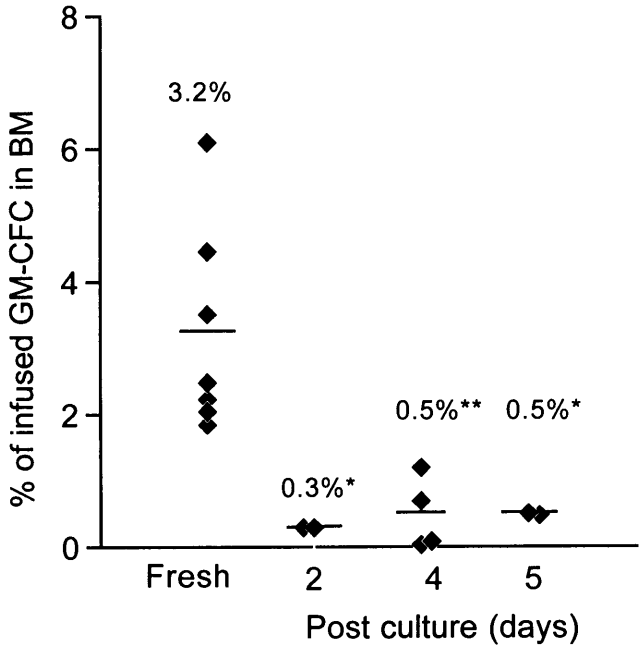


Figure 3.9 - Homing of freshly isolated and cytokine stimulated CD34$^+$ cells. Time course of changes in BM homing following cytokine activation. CD34$^+$ cells exposed to cytokines (SCF, Flt3L 100ng/ml and IL-3, IL-6 20ng/ml) for 2, 4 or 5 days were infused into sub-lethally irradiated animals and homing to BM assessed at 24hr post infusion. The homing of fresh cells is shown for comparison. The data are from 4 independent experiments, each data point represents 1 animal, and the mean values are indicated (*P<0.05, **P<0.01, ***P<0.001 compared to fresh cells, Mann-Whitney U Test)

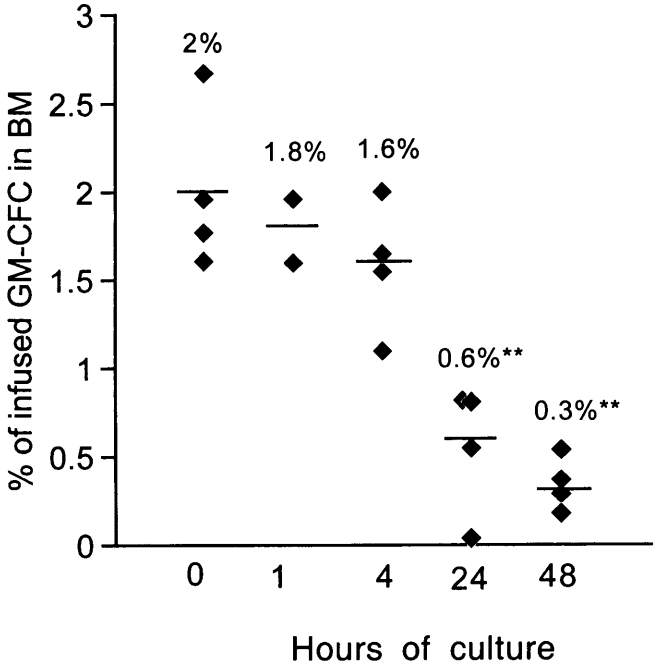


Figure 3.10 - Homing of freshly isolated and cytokine stimulated CD34$^+$ cells. CD34$^+$ cells were cultured (stimulated with SCF, Flt3L at 100ng/ml and IL-3, IL-6 at 20ng/ml) for 1, 4, 24 or 48hr and homing of infused cells to BM assessed at 24h post-transplantation, n=2 experiments, data given for each animal and the mean values are indicated (*P<0.05, **P<0.01, ***P<0.001 compared to fresh cells, Mann-Whitney U Test)

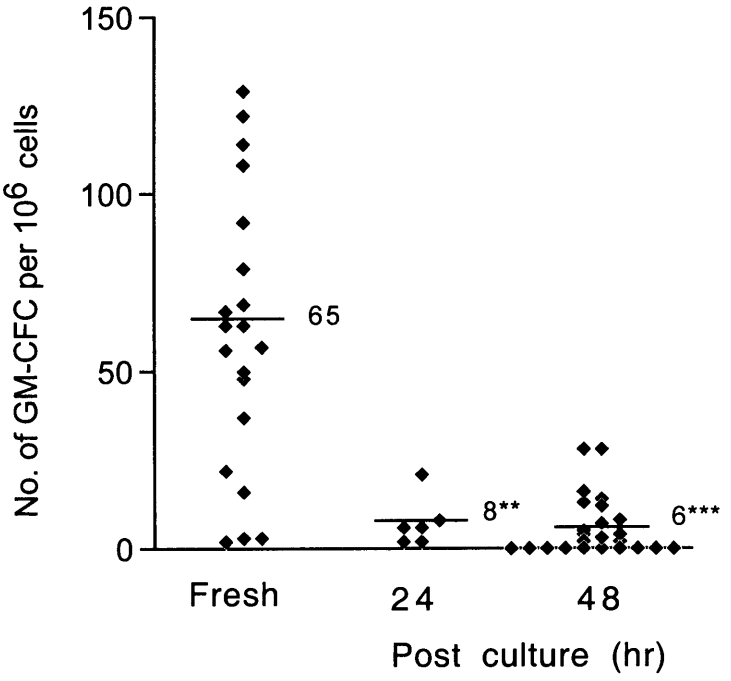


Figure 3.11 - Homing of freshly isolated and cytokine stimulated CD34$^+$ cells to the spleen. Homing of freshly isolated CD34$^+$ cells or cultured cells (stimulated with SCF, Flt3L 100ng/ml and IL-3, IL-6 20ng/ml) after 24 and 48hr of culture to the spleen at 24hr post transplantation. The homing of fresh cells is shown for comparison. The data are from 10, 3 and 8 independent experiments for Fresh, 24 and 48 hr, respectively. Each data point represents 1 animal, and the mean values are indicated (*P<0.05, **P<0.01, ***P<0.001 compared to fresh cells, Mann-Whitney U Test)

In further experiments, we used 48 hr-stimulated cells because these cells had not expanded significantly (a mean 1.1 ± 0.3 fold expansion over input numbers), n=5 (Table 3.1), so avoiding any confounding effect of infusing large numbers of cells.

3.2.7 Assessment of short term homing of transplanted CD34$^+$ cells by flow cytometric assay.

It is possible that CFC recovery of expanded human CD34$^+$ cells may be adversely affected by conditions in the irradiated murine BM. Hence I sought to confirm our results on clonogenic cells by determining the actual numbers of cells which had homed to the BM at 24 hours. To track intravenously transplanted human MPB CD34$^+$ cells, we used a well established cell labelling procedure using the fluorescent dye PKH26.

An initial experiment was carried out to determine the optimal concentration of PKH26 required to brightly stain fresh CD34$^+$ cells while maintaining cell viability. Fresh CD34$^+$ cells were stained with 2, 5, 10 and 20μM PKH26 and analysed on a flow cytometer for fluorescence intensity. Fresh unlabelled CD34$^+$ cells (viability 79%) stained with 2μM PKH26 resulted in a MFI of 7 and no loss of cell viability (82%). At a higher PKH26 concentration of 5μM cells had a 3 fold higher MFI (25) but with a fall in viability to 58%. PKH26 concentrations at 10 and 20μM showed no further increase of staining intensity (MFI of 26 and 24, respectively) but significantly affected cell viability (52% and 54%, respectively), shown in Figure 3.12. The above data show that optimal cell labelling of CD34$^+$ cells while maintaining cell viability was achieved with PKH26 at a concentration of 5μM. Therefore in all further experiments cells were labelled with PKH26 at 5μM.

Using the staining method described in Materials and Methods, 99% of MPB CD34$^+$ cells (fresh and post cultured) could be brightly stained, (Table 3.5) yielding a fluorescence intensity that is at least 1 log higher than that of unlabelled control cells. Profiles from a representative experiment of fresh and cultured cells stained with PKH26 at 5μM are shown in Figure 3.13.

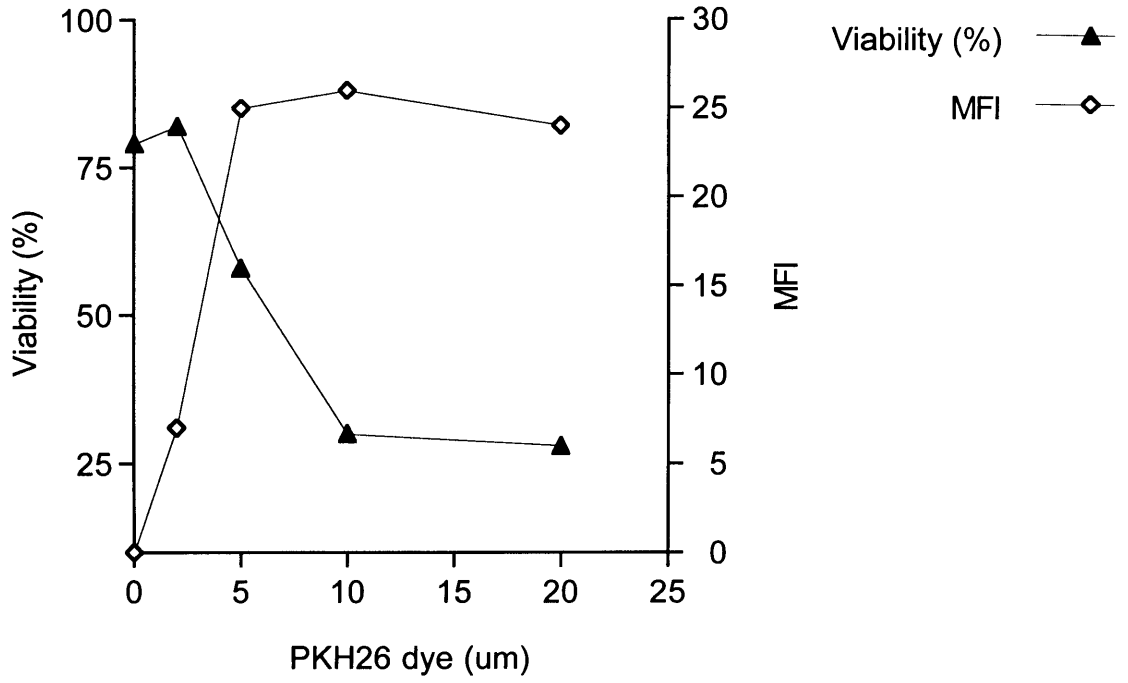


Figure 3.12 - PKH26 staining intensity and viability of MPB CD34$^+$ cells. CD34$^+$ selected PBSC progenitors were stained with PKH26 as described in Chapter 2 General Methods and were analysed for staining intensity (diamonds) and viability (triangles). Data from a representative experiment.

Table 3.5 - PKH26 labelling of $CD34^+$ selected cells, fresh and post culture

		Experiment 1*		Experiment 2^	
$CD34^+$ cells	**Staining**	**$PKH26^+$ cells (%)**	**MFI**	**$PKH26^+$ cells (%)**	**MFI**
Fresh	Control	0.4	0.1	0.1	0.1
	PKH26-labelled	99.2	56.2	100	178.8
Post Culture	Control	0.2	0.17	0.3	0.21
	PKH26-labelled	99.7	22.5	99.8	29.4

Freshly isolated $CD34^+$ or cultured cells were PKH26-labelled as described in materials and methods. Cultured cells had been stimulated with the 4 cytokine mix for 48 hours. Fluorescence intensity was assessed by flow cytometry. As control unstained cells were analysed. Data are given from two independent experiments. * Frozen thawed cells. ^ Freshly selected.

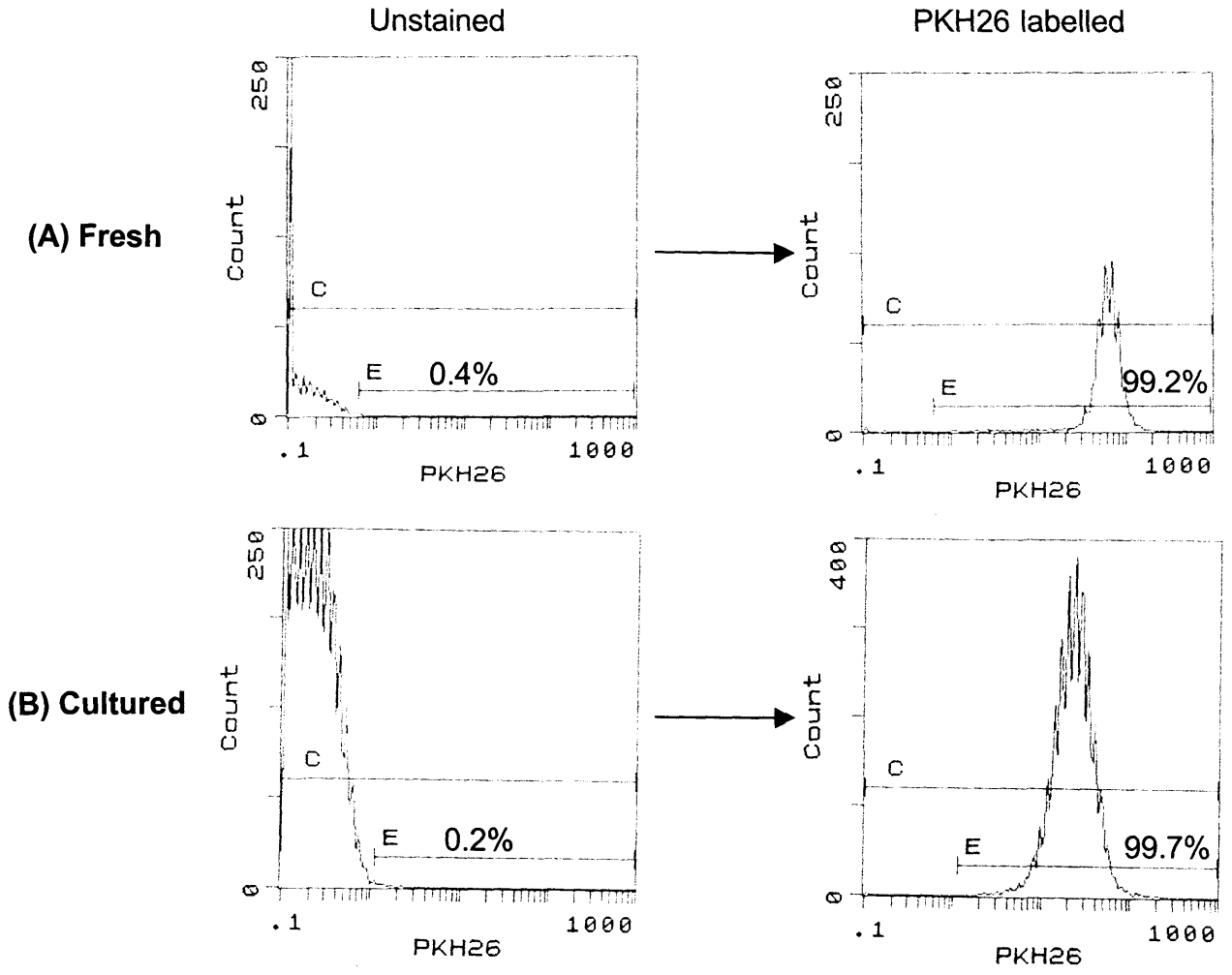


Figure 3.13 - PKH26-labelling intensity and purity of CD34⁺ selected PBSC progenitors. Fluorescence histograms showing freshly isolated MPB CD34⁺ cells (A) and CD34⁺ cells stimulated with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) in X-VIVO 10/1% HSA for 48 hr (B), unstained (left) and PKH26 stained (right) as described in Chapter 2 General Methods. Fluorescence of stained PKH26 cells was at least 1 log above that of unstained cells. A representative experiment is shown.

In order to assess PKH26 effects on CFC formation of $CD34^+$ cells, unlabeled and labelled $CD34^+$ cells were set up in the methylcellulose colony assay. As shown in Table 3.6, PKH26 labelled cells generated comparable numbers of total colonies as unstained cells, n=2. Therefore PKH26 staining did not have any deleterious effect on CFC growth assessed by methylcellulose colony assay *in vitro*. This indicates that PKH26 does not alter the functional activity of haemopoietic progenitors and is suitable to track their homing *in vivo*.

In order to evaluate the effect of cytokine exposure on homing *in vivo*, BM from mice transplanted with unlabelled and PKH26-labelled cells (fresh and post culture) was collected at 24hr post transplantation, and analysed for the presence of PKH26-labelled cells by flow cytometry. Table 3.7 shows the number of labelled cells that were detected in irradiated (Experiment 2) and non-irradiated (Experiment 1) mice per million BM cells analysed at 24hr by flow cytometry. It is clear that the short term (24hr) homing of $CD34^+$ cells was consistently reduced after cytokine exposure ($PKH26^+$ events per 10^6 BM cells were 695, 1136 and 391, 292 for each of 2 non-irradiated and 2 irradiated mice, respectively which received fresh cells vs 201, 204 and 68, 48 respectively for mice which received for 48hr cultured cells, n=2, $p<0.01$). There was a mean 5 fold reduction in homing of the cytokine-treated (48hr) cells when compared to non-cultured fresh $CD34^+$ cells. These results are presented in Table 3.7 and depicted in graphical form in Figure 3.14. As reported previously (Hendrikx *et al*, 1996), homing to non-irradiated BM was 2 to 4 times higher than to irradiated BM and this difference was observed for both fresh and cultured cells (Table 3.7). Dot plot profiles from a representative experiment are shown in Figure 3.15.

Table 3.6 - Effect of PKH26-labelling on colony forming cells (CFCs)

		No. of GM-CFCs/1000 cells	
$CD34^+$ cells	Staining	Experiment 1	Experiment 2
Fresh	Control	63	54
	PKH26-labelled	60	40
Post Culture	Control	64	58
	PKH26-labelled	63	42

Freshly isolated $CD34^+$ or cultured cells were PKH26-labelled as described in Chapter 2 General Methods. Cultured cells were stimulated with the 4 cytokine mix for 48 hours. Colony forming activity of cells was assessed in methylcellulose culture. As control, unstained cells were analysed. Data are given from two independent experiments.

Table 3.7 - Number of PKH26-labelled cells detected in NOD/SCID BM, 24hr post-transplantation.

		No. of $PKH26^+$ events/million BM cells	
$CD34^+$ cells	Staining	Experiment 1*	Experiment 2
Fresh	Control	4	2
	PKH26-labelled	695, 1136	391, 292
Post culture	Control	1	2
	PKH26-labelled	201, 204	68, 48

Freshly isolated $CD34^+$ or cultured cells (unstained and PKH26-labelled) were infused into sub-lethally irradiated NOD/SCID mice. Cultured cells were stimulated with SCF (100ng/ml), Flt3L (100 ng/ml), IL-3 (20ng/ml) and IL-6 (20 ng/ml) in X-VIVO 10 with 1% HSA for 48 hours. For both experiments homing was assessed at 24hr post transplantation by flow cytometric analysis of BM cells as detailed in Chapter 2 General Methods. Data for the homing of cells has been presented here as number of positive events per million BM cells analysed. Data are given for each animal from 2 independent experiments. * Experiment 1 mice non-irradiated.

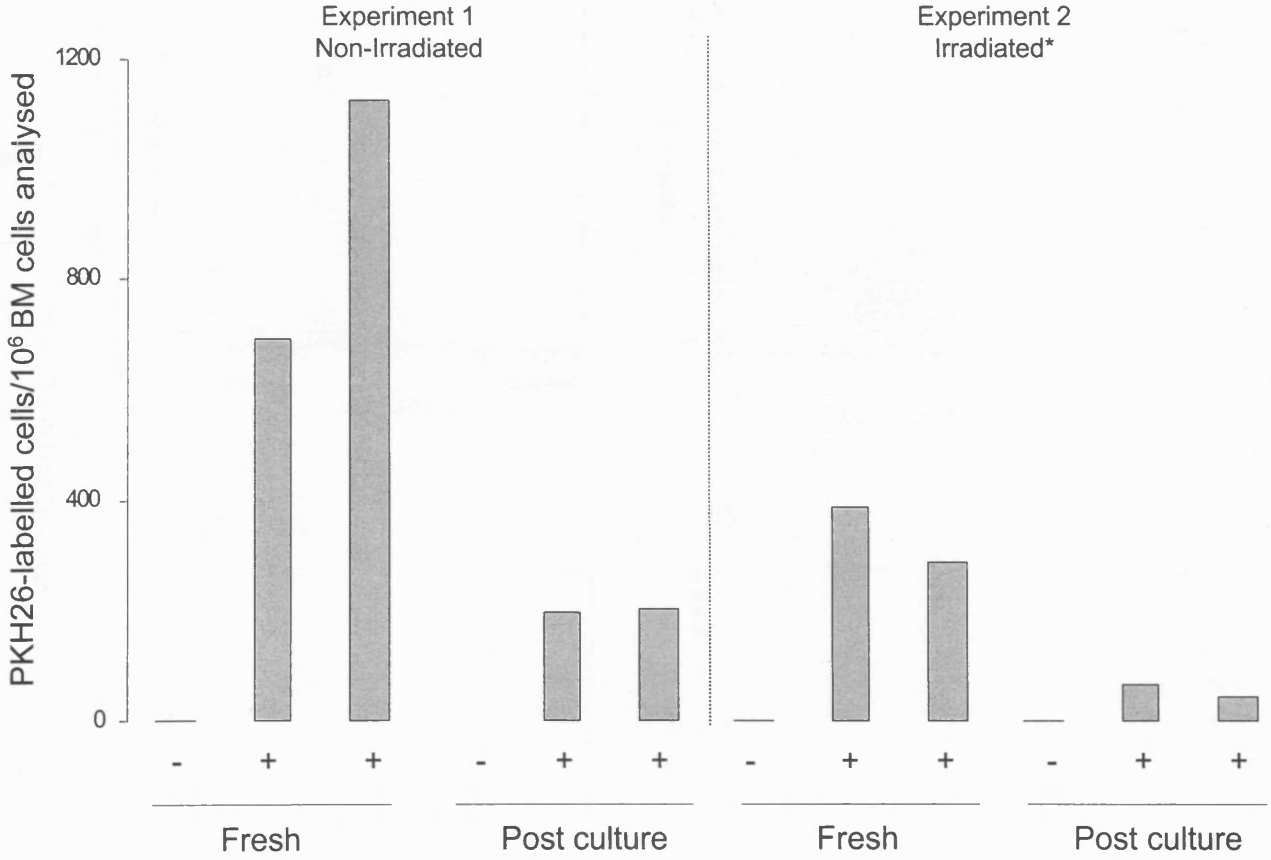


Figure 3.14 - Homing defect after 48hr cytokine exposure. To examine the effects of cytokine culture on the homing of MPB $CD34^+$ cells, animals were infused with 10 million PKH26-labelled $CD34^+$ cells (+) or unstained $CD34^+$ cells (-). 2 experiments were done each with two groups of animals; the first group received 'fresh' and the second '48hr cultured', cells. After 24hr, the animals were sacrificed and BM cells (both femurs and tibia) removed for analysis using a flow cytometer. Each bar represents an individual animal and represents number of $PKH26^+$ events/10^6 BM cells analysed. *Mice received 325rads sub-lethal irradiation immediately before injection of cells.

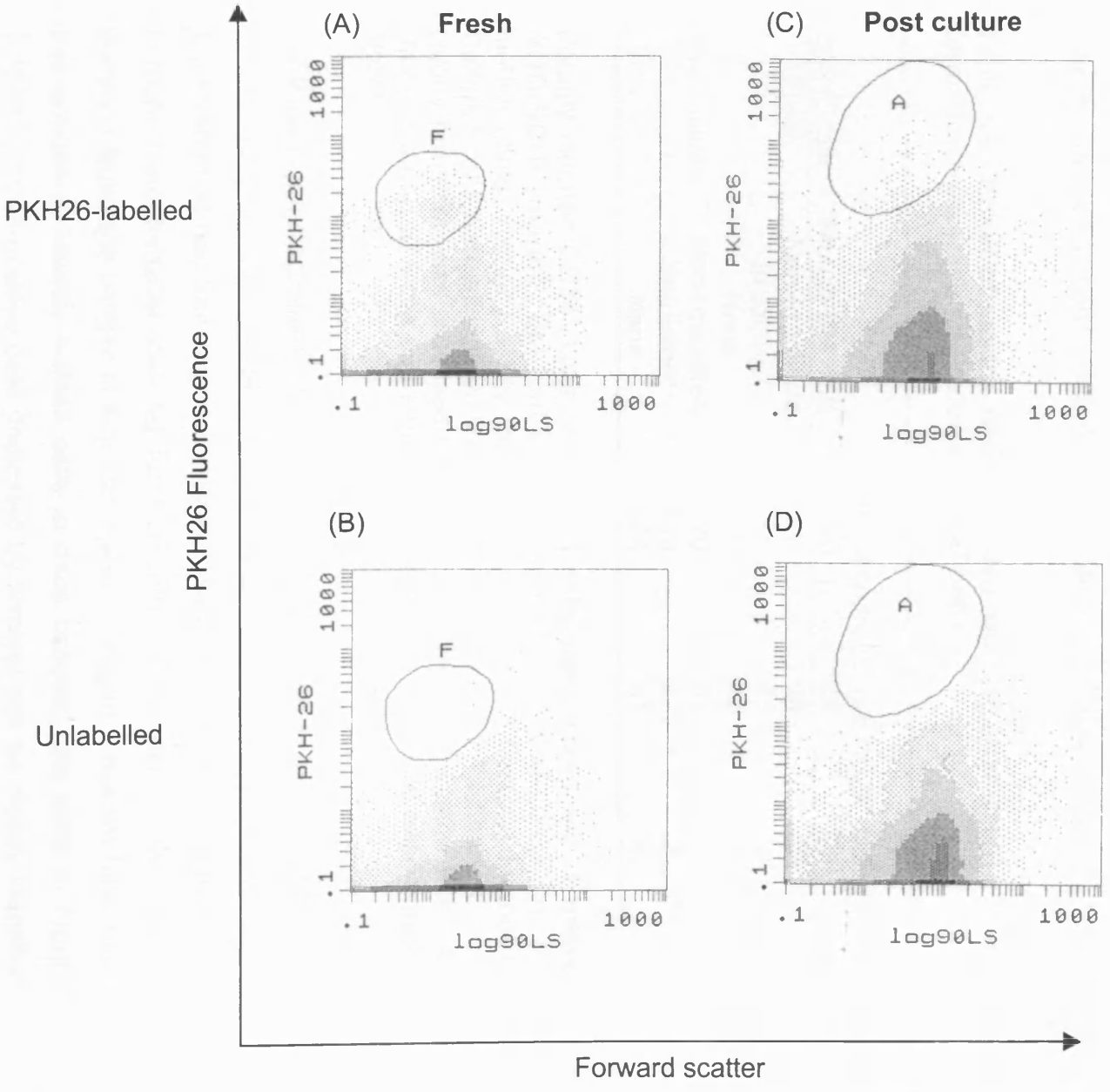


Figure 3.15 - Dot plot of PKH26 positive cells in NOD/SCID mice BM. A total of 10 million PKH26-labelled fresh (A) or 48 hr cultured (C) MPB $CD34^+$ cells were infused by tail vein into NOD/SCID mice. 24hr later, PKH26 positive cells that were present in the marrow were identified by flow cytometry. PKH+ve events are indicated by gates. Gate F (1763 events) for the animal which received fresh cells (A) contains more cells than Gate A (227 events) for the animal which received cultured cells. Animals infused with 10 million unlabelled cells (B,D) were analysed as a control. In these animals, events contained with Gates F and A were negligible (1 and 6 respectively). Profiles from a representative experiment.

3.2.8 Effect of irradiation on murine BM cells and CFC formation

Mice that have been sub-lethally irradiated few hours before transplantation may produce factors (TGF-β and MIP1-α) that suppress colony formation (the so called 'cytokine storm' observed after radiation). In order to exclude the possible suppression of colony formation by negative cytokines released by irradiated murine BM cells, both fresh and expanded human $CD34^+$ cells were cultured in the presence of a large number of BM cells (1 million) from irradiated and non-irradiated animals. The number of assayable progenitors was determined by methylcellulose colony assay. There was no difference in the number of CFC produced by fresh and expanded cells in the presence of 1 million irradiated or non-irradiated murine BM cells (Table 3.8).

Table 3.8 - Effect of murine BM (irradiated and non-irradiated) on CFC formation from fresh and cultured $CD34^+$ cells.

		No. of GM/CFC per $CD34^+$ cells		
$CD34^+$ cells	Mouse BM cells	1000	500	250
Fresh	Non-Irradiated	81	49	31
	Irradiated*	73	45	24
	None	66	49	20
Post culture	Non-Irradiated	120	67	38
	Irradiated*	118	42	27
	None	125	51	42

Freshly isolated $CD34^+$ cells or cultured cells were mixed with 1 million NOD/SCID murine BM cells and assayed for GM-CFCs in the methylcellulose colony assay (described in Chapter 2 General Methods). Cultured cells were stimulated with the 4 cytokine mix for 72 hours. Control colony formation was assessed in the absence (None) of murine BM cells. *Mice received 375rads sub-lethal irradiation. Data from one experiment is shown.

3.2.9 Immunohistochemical identification of human cells in mice tissues.

In order to confirm that $CD34^+$ cells identified in murine tissues at 24hr post-transplantation had transmigrated into extravascular tissues, we carried out immuno-histochemical staining for CD45RB. CD45 also known as the common leukocyte antigen is a human specific antigen. Thus we have used this antigen to identify human cells in mice tissues. As seen in Figure 3.17A&B, CD45-positive cells (indicated by arrows) can be clearly identified

amongst murine haemopoietic tissue outside the bone marrow sinuses. BM sections from animals that had received cytokine activated cells contained far fewer $CD45^+$ cells at 24 hours post-transplantation, when compared with animals that had received fresh cells. Analysis of BM from animals at 6 weeks post-transplantation shows the presence of numerous $CD45^+$ cells, confirming haemopoietic engraftment (Figure 3.16). The human tonsil sections served as positive control for $CD45^+$ cells (Figure 3.16). Immuno-histochemical analysis of lung sections at 24hr post-transplantation (Figure 3.17B) confirmed the presence of $CD45^+$ cells. BM sections from animals infused with 0.5ml of saline served as a negative control (Figure 3.17A). BM section from mice infused with saline showed no positive staining (Figure 3.17A) as expected.

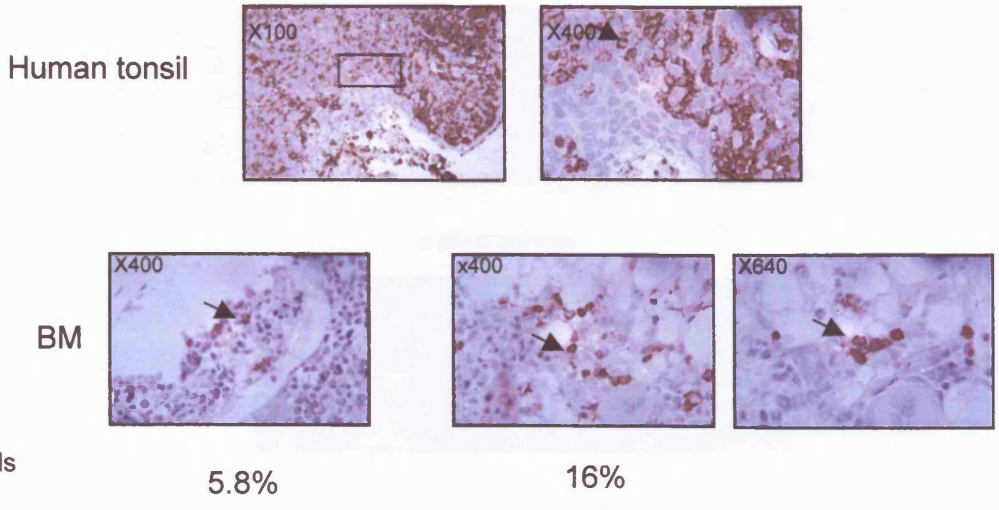


Figure 3.16. Immunohistochemical identification of human CD45$^+$ cells in the BM of NOD/SCID mice at 6 weeks following transplantation. Sections of BM of mice that received 1-2x10^6 freshly isolated CD34$^+$ cells by tail vein injection were analysed for CD45RB expression at 6 weeks post transplantation. Sections from BM of a representative experiment are shown. Human cells (CD45$^+$) in murine tissue are indicated by arrows and a dark brown staining. Numbers indicate the percentage of CD45$^+$ cells in the BM by FACS analysis. Cells were viewed with the magnifications indicated in each plate. Human tonsil sections served as positive control.

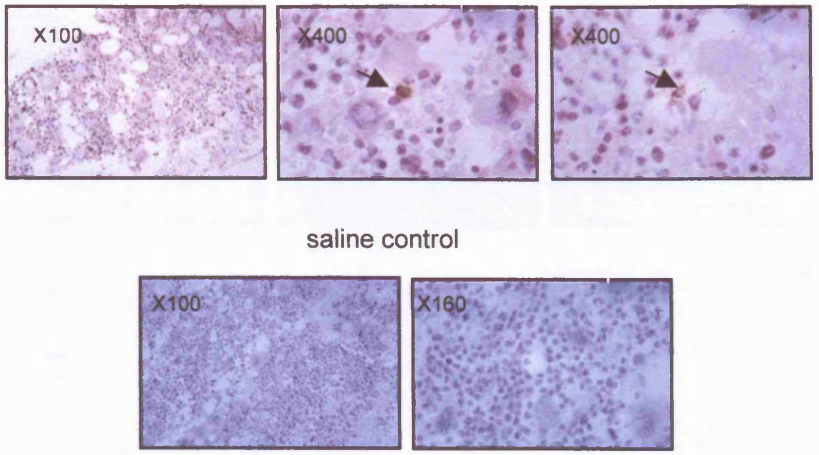


Figure 3.17A. Immunohistochemical identification of human CD45$^+$ cells in the BM of NOD/SCID mice at 24 hr following transplantation. Sections of BM of mice that received 1-2x10^6 freshly isolated CD34$^+$ cells by tail vein injection were analysed for CD45RB expression at 24hr post transplantation. Saline control mice were infused with HBSS and served as negative control. Sections from BM of a representative experiment are shown. Human cells (CD45$^+$) in murine tissue are indicated by arrows and a dark brown staining. Human tonsil sections served as positive control. Cells were viewed with the magnifications indicated in each plate.

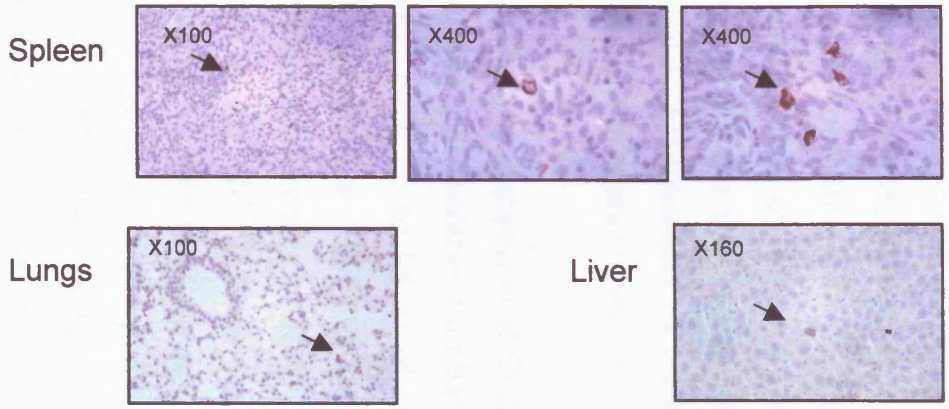


Figure 3.17B. Immunohistochemical identification of human CD45$^+$ cells in the spleen, lungs and livers of NOD/SCID mice following transplantation. Sections of spleens, lungs and liver of mice that received 1-2x10^6 freshly isolated CD34$^+$ cells by tail vein injection were analysed for CD45RB expression at 24hr post transplantation. Saline control mice were infused with HBSS and served as negative control. Sections from spleen, lungs and liver of a representative experiment are shown. Human cells (CD45$^+$) in murine tissue are indicated by arrows and a dark brown staining. Human tonsil sections served as positive control. Cells were viewed with the magnifications indicated in each plate.

3.3 DISCUSSION

$CD34^+$ cells cultured with the cytokine combination of SCF, Flt3L, IL-3 and IL-6 demonstrate a rapid expansion in total cell number. This particular cytokine combination has been optimised for support and maintenance of primitive HSCs in culture (Conneally *et al*, 1997;Dao *et al*, 1997) and used for retroviral transduction of $CD34^+$ cells (Demaison *et al*, 2000). With this cytokine combination at 48hr of culture cells had not expanded significantly, but by day 3 had expanded 4 fold. Similar kinetics have been reported using both UCB $CD34^+$ cells (Dorrell *et al*, 2000) and MPB $CD34^+$ cells (Danet *et al*, 2001). In the former study, the cytokine combination of IL-6, G-CSF, SCF, Flt3L was used to culture cells for 1,2,3,4 and 6 days. During the culture period I performed with the cytokine combination of SCF, Flt3L, IL-3 and IL-6, a change in cell morphology was also observed. Freshly isolated $CD34^+$ cells appeared primitive with a high nuclear to cytoplasmic ratio (scanty cytoplasm), but by 48hr of culture, displayed a decreased nuclear to cytoplasmic ratio with a basophilic cytoplasm. The presence of primary azurophilic granules and vacuoles were seen with the odd mitotic cell. This is in line with the typical morphological features of cells cultured *ex vivo*.

Regarding the expansion of CFCs, cytokine expanded cells in liquid culture generated comparable numbers of total GM-CFCs colonies as freshly isolated cells up to 96 hr of culture. A similar finding was reported by (Guenechea *et al*, 1999) using UCB cells. A slightly different finding was reported earlier by (Danet *et al*, 2001) using MPB $CD34^+$ cells cultured in the presence of SCF, TPO and Flt3L without serum. They evaluated the clonogenic activity (CFC) every 24 hr for 4 days and found that the first 2 days of culture were characterised by an increase in frequency and total number of CFC. From Day 2 to 4, the combination of a decreasing CFC frequency and increasing cell number resulted in the maintenance of the total number of CFC.

Immunophenotypic analysis of the *ex vivo* expanded $CD34^+$ cells showed no significant changes in the expression of the early haemopoietic marker CD34 and also of CD38, indicating that the cells have maintained a primitive

phenotype within this short culture period (48 hour). The β1 integrins VLA-4 and VLA-5, key adhesion molecules considered to be important for anchorage and migration into the BM tissue (Papayannopoulou *et al*, 1995;Papayannopoulou *et al*, 2001;Orschell-Traycoff *et al*, 2000;Giet *et al*, 2002) were upregulated upon cytokine exposure. A similar finding was also reported by (Young *et al*, 2001) using human MPB $CD34^+$ cells stimulated in X-VIVO 15 serum free medium containing 1% BSA with c-kit ligand (100ng/ml), Flt3 ligand (100ng/ml) and TPO (50ng/ml). In contrast (Szilvassy *et al*, 2001b) found that *ex vivo* expansion of murine haemopoietic cells led to a downregulation of β1 integrin expression. Such different observations may be due to species-related differences in the regulation of integrin expression in HSPC (Szilvassy *et al*, 2001b). The increased expression of VLA-4 and VLA-5 on cultured human MPB $CD34^+$ cells may lead to a greater ability for cultured cells to adhere and migrate compared to non cultured fresh cells. In contrast there was no change in expression of PECAM-1, an adhesion molecule involved in the transmigration of $CD34^+$ cells across endothelium (Yong *et al*, 1998). PECAM-1 (also known as CD31) is a member of the Ig superfamily and is expressed by $CD34^+$ cells at high levels, with little difference in expression between freshly isolated and growth factor activated cells (Yong *et al*, 1998).

Another important receptor for homing and engraftment of transplanted HSPC is CXCR4, which binds the chemokine SDF-1 (see section 1.7.2.3). Interestingly, freshly isolated MPB $CD34^+$ cells express very low levels (3%) of the chemokine receptor CXCR4, however upon cytokine stimulation the levels rise dramatically to 51%. Peled *et al.,* (1999b) also reported that stimulation (24-48hr) of MPB $CD34^+$ cells with SCF resulted in increased CXCR4 expression (Peled *et al*, 1999b). The key role played by the chemokine receptor CXCR4 in homing and engraftment of $CD34^+$ cells was demonstrated by use of a blocking antibody to CXCR4 (Peled *et al*, 1999b;Lapidot & Kollet, 2002). These workers found that pre-treatment of human cells (UCB $CD34^+$, MPB $CD34^+$ and BM $CD34^+$ cells) with antibodies to CXCR4 prevented engraftment in NOD/SCID mice. Thus cytokine

exposure results in cells displaying a phenotype with potentially a greater adhesive and migratory potential. Yet such cells display reduced homing to the BM *in vivo* and reduced engraftment. In a recent report by (Kahn *et al*, 2004) they observed that both CB and MPB $CD34^+$ cells overexpressing CXCR4 (lentiviral gene transfer technique) demonstrated at least 2-fold increase in homing to the spleen of NOD/SCID mice; however, this effect was not observed in the BM (Kahn *et al*, 2004). Finally, short term culture induced expression of Fas/CD95 on cultured cells. Freshly isolated $CD34^+$ cells express very low levels of Fas (6%) but after 2 days of culture, Fas expression increased substantially to 65%. This has been reported previously on MPB $CD34^+$ cells (Young *et al*, 2001) and UCB $CD34^+$ cells (Liu *et al*, 2003). Increased Fas expression on cultured cells could result in increased susceptibility to apoptosis *in vivo* when encountering Fas ligand (FasL) e.g. on surface of endothelium (Josefsen *et al*, 1999).

The detrimental effects of cytokine stimulation on murine and human HSPC engraftment have been known for many years. Most of the evidence comes from studies carried out in animal models (Guenechea *et al*, 1999;Peters *et al*, 1996;van der Loo & Ploemacher, 1995;Szilvassy *et al*, 1999;Liu *et al*, 2003;Young *et al*, 2001). Young and co-workers (Young *et al*, 2001) demonstrated that NOD/SCID mice receiving uncultured MPB $CD34^+$ cells had a mean of 20% $CD45^+$ (human) cells in their BM 6 weeks post transplantation, versus 3% for mice receiving cells cultured for 3-5 days in KL, FL and TPO. I confirmed that cytokine stimulation of human MPB $CD34^+$ cells compromises engraftment potential. MPB $CD34^+$ cells cultured in serum free medium with the cytokines SCF, Flt3L, IL-3 and IL-6 have a reduced engraftment *in vivo*, which is evident by 48 hr of culture and is unaltered up to 5 days of culture. The cytokine induced engraftment defect was observed regardless of whether the expanded equivalent or equal numbers of cultured $CD34^+$ cells were infused. This excluded the possible dilutional effect of cytokine expansion on progenitor numbers. Multi-lineage engraftment was demonstrated for both fresh and post cultured cells, an observation confirmed by (Guenechea *et al*, 1999), the only difference being that the predominant lymphoid over myeloid engraftment by fresh $CD34^+$ cells is not

seen with post culture cells. This confirms an earlier observation by (Blundell *et al*, 1999). As outlined above cultured $CD34^+$ cells have increased levels of adhesion molecules (VLA-4 and VLA-5) and the chemokine receptor CXCR4 shown to be important in homing and engraftment (Peled *et al*, 1999b;Peled *et al*, 2000), but this is in contradiction with the reduced engraftment seen *in vivo*. One explanation for the reduced engraftment of cultured HSCs may be that the changes in surface receptor expression outlined above lead to increased cell migration to non-haemopoietic organs where clearance and destruction of these cells occurs (Szilvassy *et al*, 1999;Papayannopoulou & Craddock, 1997). This would thus alter stem/progenitor cell homing properties by reducing the localisation of cells into the BM. The increased localisation of cultured cells to the lungs of animals would be in accord with this hypothesis (Figure 3.8C).

I investigated the homing ability of fresh vs cultured $CD34^+$ cells using firstly the CFC surrogate assay readout. *Ex vivo* culture had a striking effect on the homing behaviour of $CD34^+$ cells, resulting in a marked fall in homing to the BM, with a concomitant increase in cells localising to the lungs. Reduced homing to the BM was evident by 24hr and persisted for up to 5 days, with no sign of reversibility. Reduced homing to the spleen was also observed at 24 and 48hr of culture. Thus, the defect in homing and engraftment occurred rapidly following $CD34^+$ cells exposure to cytokines at a time when the majority of the cells would not have divided (Yong *et al*, 2002b). Yong *et al.,* (2002b) used the cytoplasmic dye, CFSE to track the divisional history of cytokine-activated $CD34^+$ cells and demonstrated that approximately 80% and 70% of cells remained undivided at days 1 and 3 post stimulation, respectively (Yong *et al*, 2002b). This rapid and sustained loss of engraftment potential in adult $CD34^+$ cells after cytokine exposure is in accord with another published study, however in that study the authors did not follow in parallel the changes in homing behaviour (Young *et al*, 2001). Levels of homing in my study on freshly isolated MPB $CD34^+$ cells are similar to the findings of van Hennik and co-workers, who quantified human CFC and CAFC at 22-24 hours after injection into irradiated NOD/SCID animals (van Hennik *et al*, 1999). The rapid time course of the homing process is also

in accord with other reports using cord blood or adult blood $CD34^+$ cells (Kerre *et al*, 2001;Kollet *et al*, 2001). Kollet and co-investigators (Kollet *et al*, 2001) found that 48 hour stimulation of cord blood $CD34^+$ cells with SCF and IL-6 increased the numbers of CFC recovered from the BM of transplanted animals, but did not quantify these as a percentage of infused cells, hence no information regarding the efficiency of the homing process is available.

Thus I have demonstrated the defective homing of cytokine stimulated human adult $CD34^+$ cells using a functional assay for CFC to assess human progenitor cells in the BM. In support of the functional assay to quantify the homing of human HSPC, Cashman and Eaves (Cashman & Eaves, 2000) in a systematic study of the homing behaviour of functionally defined subsets of human $CD34^+$ cells have recently reported that the homing of repopulating units, CAFC and CFC are very comparable, ranging from 3-6%.

Nevertheless, the functional assay for CFC may be influenced by factors produced by the murine BM following irradiation (Cohen *et al*, 2000;Ferrara, 1993). This may adversely affect the growth of cytokine activated $CD34^+$ cells. I have shown however, that there was no difference in the number of assayable human progenitors in the presence of irradiated and non-irradiated murine BM cells, thus excluding a role for inhibitory factors on colony formation. These results indicate once again that irradiated murine BM cells do not preferentially affect cultured cells and that the impaired homing observed with cultured cells is not due to any inhibitory factor released by the irradiated BM.

The defective homing of cytokine stimulated human adult $CD34^+$ cells was also confirmed using a cell tracking assay. Short term (24hr) homing of PKH26 labelled $CD34^+$ cells was consistently reduced after 48 hr of culture in cytokines, with a mean 5 fold reduction in homing of the cytokine treated cells when compared to non-cultured fresh $CD34^+$ cells. This reduction in homing has also been documented for murine stem cells using a similar assay. Cerny *et al.,* (2002) used purified Lin^- $Sca\text{-}1^+$ murine BM cells stained with the dye CFSE and reported defective homing of these cells following culture

for 48 hr (cytokines IL-3, IL-6, IL-11 and Steel factor) when compared to non-cultured cells (Cerny *et al*, 2002;Dooner *et al*, 2004). An additional finding in my studies was that homing of both fresh and cultured cells to non-irradiated BM was 2-4 times higher than to irradiated BM, a finding reported earlier by (Hendrikx *et al*, 1996) for murine stem cells labelled with the dye PKH26. This effect of the irradiated murine BM appears to affect both fresh and cultured cells equally.

Immuno-histochemical staining of BM and spleen section for CD45RB confirmed that $CD34^+$ cells identified in murine tissues at 24 hour post transplantation had transmigrated into extravasacular tissues. The staining of BM and spleen sections confirmed the presence of CD45 positive cells in the extravascular tissues. BM sections from animals that had received cytokine activated cells contained far fewer $CD45^+$ cells at 24 hr post transplantation, when compared to animals that had received fresh cells. BM from engrafted animals showed the presence of numerous $CD45^+$ cells, confirming haemopoietic engraftment.

Recently, there has been speculation that cytokine stimulation induces changes in adhesion molecule expression of HSCs resulting in decreased localisation of these cells to the BM, and increased accumulation in non-haemopoietic organs where clearance and destruction of these cells occurs (Dooner *et al*, 2004;Moore, 2002). The data I present here are in agreement with the hypothesis that the reduced engraftment documented previously with cultured cells may be due to the reduced homing of cells to the BM as a result of cells lodging in the lungs. The question still remains whether the cells are trapped in the lungs and therefore are unable to get into the BM, or if they unable to get into the BM because they have increased ability to migrate into non-haemopoietic tissue like the lungs.

In summary, I have demonstrated in adult MPB $CD34^+$ cells that cytokine exposure *ex vivo* leads to a loss of engraftment ability *in vivo* which occurs rapidly and which is associated with a striking alteration in the tissue

distribution of homed cells. Loss of homing to the BM and to a lesser extent, the spleen, coincides with increased accumulation of cells in the lungs.

Chapter 4: Influence of cell cycle status on homing of MPB $CD34^+$ cells

4.1 INTRODUCTION

Influence of cell cycle phase on engraftment

Exposure of stem cells to growth factors *in vitro* reduces the *in vivo* repopulating ability of these cells, an effect which has been found to precede execution of a first cell division. Previous studies suggest that when human HSPC are stimulated to proliferate *in vitro*, there is a loss of engraftment ability as cells progress into active phases of the cell cycle (Glimm *et al*, 2000;Orschell-Traycoff *et al*, 2000;Szilvassy *et al*, 2000;Gothot *et al*, 1997). Gothot *et al.,* (1998) showed in freshly harvested populations of human MPB $CD34^+$ cells, that NOD/SCID repopulating cells reside in G_0 rather than G_1 (Gothot *et al*, 1998). Following culture for 36 hr with IL-3, KL, and FL, $CD34^+$ cells moved from G_0 to G_1 and their repopulating capacity markedly diminished with the transit into G_1. Thus, both G_0-G_1 progression and entry into S/G_2/M *in vitro* are associated with a decrease in HSC engraftment capacity. A study using highly purified murine stem cells found this engraftment defect to be reversible, and to oscillate as cells undergo synchronous cell cycle passage with engraftment nadirs in late S-and early G_2-phases of the cell cycle (Habibian *et al*, 1998). In murine lin^- BM cells cultured in FL, SCF, IL-11, repopulating capability fell dramatically just prior to cell division, but recovered 24 hours later at which time the repopulating cells were recovered in the divided fraction (Oostendorp *et al*, 2000). *Ex vivo* culture of murine bone marrow cells results in an engraftment defect concomitantly with progression of HSC through S phase, suggesting that cell cycle transit impairs their ability to engraft (Kittler *et al*, 1997). Based on these data, it has been hypothesised that position in the cell cycle may determine whether long term engraftment will occur and that certain phases of the cell cycle may be incompatible with the multistep pathway of homing and proliferation of self-renewing HSC (Moore, 2002). The hypothesis is that actively cycling stem/progenitor cells are unable to "home" to haemopoietic tissue, this homing defect being especially pronounced for cells in S/G_2/M. Support for this hypothesis derives from murine studies where seeding of HPC/CFC to the BM/spleen was found to decrease following *ex vivo* cytokine exposure (Szilvassy *et al*, 1999;van der Loo & Ploemacher, 1995).

In vitro migration of G_0/G_1 and $S+G_2/M$ fractions

In vitro studies in homing have focussed on the early critical step of transendothelial migration, whereby HSPC egress from the circulation into haemopoietic tissue. Yong *et al.,* (1998) have shown that while $CD34^+$ cells adhere readily to the endothelial surface, they do not undergo transmigration unless activated by cytokines that induce cell division (Yong *et al*, 1998). The former group also showed that the migratory capacity is selectively greater for cells which are in G_0G_1 phase of the cell cycle (Yong *et al*, 2002b), a finding recently confirmed by other workers (Giet *et al*, 2002). Although these observations provide a possible basis for the cell cycle dependency of engraftment, workers have yet to obtain direct confirmation that the defective engraftment of cytokine cultured cells occurs as a result of reduced homing ability of actively cycling cells.

Modulating cell cycle progression and effects on engraftment

If actively cycling HSPC have an engraftment defect, then modulating cell cycle progression to induce quiescence at time of infusion might restore the repopulating potential. Exposure to TGF-β increased the proportion of HSPC in G_0G_1 phase of the cell cycle, but did not aid engraftment in either a murine model or xenogeneic model (Wiesmann *et al*, 2000;Glimm *et al*, 2000). In a recent study, preactivated primate $CD34^+$ cells were "rested" for 2 days in SCF and retronectin, resulting in an increase in the proportion of cells in G_0G_1 and in superior engraftment compared with control 'non-rested' cells (Takatoku *et al*, 2001). SDF-1 has recently been reported to enhance survival, while inhibiting cycling of HSPC (Cashman *et al*, 2002;Glimm *et al*, 2002). Incubation of proliferating cord blood $CD34^+$ cells with SDF and TPO produced an increase in CRU frequency, although without any changes in the cell cycle profile of the cells (Glimm *et al*, 2002). Intriguingly, there is now evidence that the cell cycle constraints on engraftment may not apply to HSC from early ontogeny. Foetal liver $CD34^+$ cells, whether in G_0, G_1 or $S/G_2/M$ are able to engraft immunodeficient animals (Wilpshaar *et al*, 2002). Although there may be ontogeny-related differences in the homing and engraftment behaviour of HSC from different sources, this finding raises the

possibility that the engraftment defect of cultured cells is the result of *ex vivo* cytokine activation and is not directly related to cell cycling per se.

Chapter Aims

The observed engraftment defect of proliferating human HSC during passage through $S/G_2/M$ phases of the cell cycle and the failure to re-enter G_0 may have a considerable negative impact on the clinical utility of current *ex vivo* expansion or retroviral marking protocols dependent on maximal induction of stem cell cycling (Moore, 2002). The present study was designed to determine if alterations in the homing and engraftment of cytokine stimulated MPB $CD34^+$ cells are cell cycle dependent. I investigated the cell cycle dependence of the homing and engraftment process by using sorted cells in G_0G_1 and $S/G_2/M$ phases of the cell cycle, and also by inducing cell cycle arrest at the G_1/S border, followed by synchronised S phase progression.

4.2 Special Methods

4.2.1 Cell synchronisation using aphidicolin

$CD34^+$ cells were stimulated with SCF, Flt3L and TPO at 100ng/ml in X-VIVO 10 /1%HSA at a cell density of $0.5x10^6$/ml in tissue culture flasks (Nalge Nunc) at 37°C and 5% CO_2. After 16 hours, aphidicolin (2µg/ml, Sigma) was added and incubation continued for a further 24 hours. At the end of this incubation (40 hours altogether), cells were centrifuged at 1800 rpm for 10 minutes and washed with 10ml X-VIVO 10/1% HSA. After aliquots were removed for cell cycle analysis, cells were re-cultured in X-VIVO 10/1% HSA with cytokines (SCF, Flt3L and TPO at 100ng/ml) at $0.5x10^6$/ml in order to allow S phase progression. Cells were harvested at 3 and 6 hours thereafter to assess their position in the cell cycle (see section 2.2.8) and their homing ability.

4.2.2 Cell cycle fractionation with Hoechst 33342.

$CD34^+$ cells were cultured in X-VIVO 10/1% HSA supplemented with SCF, Flt3L (100ng/ml) IL-3, IL-6 (20ng/ml) at 37°C and 5% CO_2 for 48 hr. Cultured cells were washed once in 10ml of Hst buffer (HBSS, 20mM HEPES, 1g/l glucose, 10% FCS) and resuspended at $5x10^6$/ml in 10µM solution of

Hoechst (Hst) 33342 (Molecular probes) in Hst buffer. After incubation at 37°C in a water bath for 45 minutes cells were incubated for another 45 minutes at 37°C with Pyronin Y (2.5μg/ml, Sigma). Cells were washed once in 10ml of chilled Hst buffer, resupended in a maximum of 1ml Hst buffer, transferred to a sterile capped test tube and sorted on a FACS vantage equipped with a multiline ultraviolet laser (351-364nm) providing excitation for Hst. Hst signal was detected with a 424 ± 22nm band pass filter. Sorting windows were constructed to sort G_0/G_1 from $S/G_2/M$ populations. Cells were kept on ice during sorting to minimise dye leaking and were protected from light. No effect of Hst and Py staining on colony formation was detected (Table 4.2). Post sort analysis was performed on sorted cells with the use of initial instrument settings employed during sorting. Purity of sorted G_0/G_1 cells exceeded 98% and of $S/G_2/M$ cells exceeded 83%.

4.3 RESULTS

4.3.1 Short term BM homing of sorted G_0/G_1 and $S+G_2/M$ cells

4.3.1.1 Human MPB $CD34^+$ cells placed in liquid culture rapidly enter cell cycle

$CD34^+$ cells were cultured in X-VIVO 10/1% HSA supplemented with the 4 cytokine mixture. Culture under these conditions increased the proportion of actively cycling cells from 3±1% to 28±3% in $S/G_2/M$ after 48 hours (Figure 4.1). As shown in Figure 4.1, 96±1% of freshly selected $CD34^+$ cells were in G_0/G_1 whereas by 24 and 48 hr of culture this proportion had decreased to 81±9% and 72±2%, respectively, n=3. Representative cell cycle profiles of freshly selected $CD34^+$ cells (Figure 4.2Ai) and cells after 48hr post stimulation (Figure 4.2Aii) are shown in Figure 4.2A. The time course of the observed changes in BM homing (Chapter 3, Figure 3.10) closely follows the kinetics of cells entering into the active phase of the cell cycle (Figure 4.1), suggesting that cytokine induced changes in BM homing may relate to position in cell cycle. To directly address this question, I proceeded to sort cytokine stimulated $CD34^+$ cells on basis of cell cycle status.

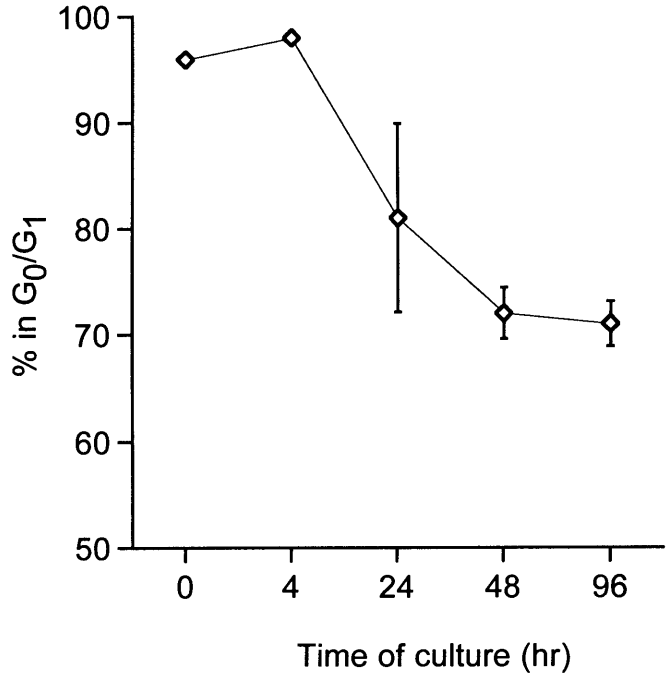


Figure 4.1 - Cell cycle analysis of freshly isolated MPB CD34$^+$ cells and cytokine stimulated cells. Freshly isolated CD34$^+$ cells were cultured with SCF, Flt3L at 100ng/ml and IL-3, IL-6 at 20ng/ml as detailed in Chapter 2 General Methods and aliquots removed for cell cycle analysis at time points indicated. Cells were fixed and stained with PI and analysed on an Epics-Elite flow cytometer (Coulter Electronics). The percentages of cells within G_0/G_1 phase of the cell cycle are shown. The data are the mean ± SEM of 3 experiments, except time point 4hr n=1.

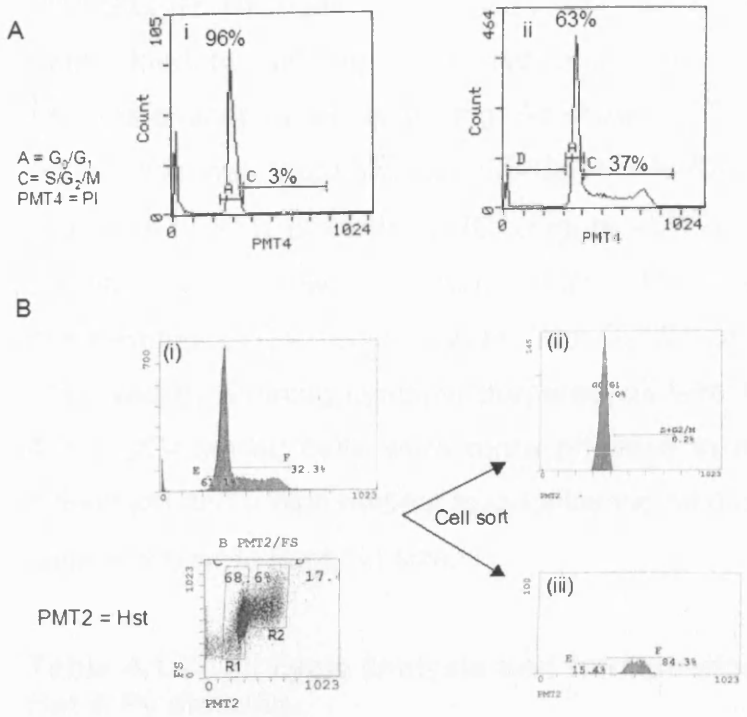


Figure 4.2 - Cell cycle fractionation of cytokine stimulated CD34$^+$ cells. Cell cycle analysis of MPB CD34$^+$ cells by PI (A) and fractionation using Hst and Py Y staining (B). (A) DNA histograms of MPB CD34$^+$ cells immediately after isolation (i) and 48hr post culture (ii) by PI staining. (B) Cultured CD34$^+$ cells were simultaneously stained with Hst and Py Y, the flow histogram shows gates indicating G_0/G_1 (R1) and $S/G_2/M$ (R2) populations of MPB CD34$^+$ cells (i). Sort windows to collect G_0/G_1 and $S/G_2/M$ cells are indicated as regions R1 and R2, respectively. The purity of sorted G_0/G_1 cells (ii) exceeded 98% and $S/G_2/M$ cells (iii) exceeded 83%. A representative experiment is shown.

4.3.1.2 Cell cycle fractionation of cytokine stimulated CD34$^+$ cells

CD34$^+$ cells were cultured in X-VIVO 10/1% HSA supplemented with the 4 cytokine mixture for 48 hr, stained with Hst/Pyronin Y and sorted into G_0/G_1 and $S/G_2/M$ fractions (Figure 4.2). The purity of sorted populations was determined by running the sorted cells with the use of initial instrument settings employed during sorting. As shown in Table 4.1, the purity of G_0/G_1 sorted cells was 98±0.3% and of $S/G_2/M$ cells 83±4.7%, n=4. Representative cell cycle profiles of sorted G_0/G_1 (Figure 4.2Bii) and $S/G_2/M$ (Figure 4.2Biii) fractions are shown in Figure 4.2B. The morphological features of proliferating CD34$^+$ cells sorted into G_0/G_1 and $S/G_2/M$ fractions were observed by staining cytospin preparations with MGG, as shown in Figure 4.3. G_0/G_1 sorted cells were more primitive in appearance with a scanty cytoplasm and a high nuclear to cytoplasmic ratio compared to $S/G_2/M$ sorted cells which were larger in size.

Table 4.1 - Cell cycle analysis and fractionation of MPB CD34$^+$ cells by Hst & Py staining.

	Population	% of cells (mean ± SEM)
Presort	G_0/G_1	67.6 ± 3
	$S+G_2/M$	31.3 ± 3.9
Post sort	G_0/G_1	98.4 ± 0.3
	$S+G_2/M$	83.6 ± 4.7

CD34$^+$ cells were stimulated with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) in X-VIVO 10/1% HSA for 48hr (presort). Cultured cells were stained with Hst and Py Y as described in Chapter 4 Special Methods and sorted for G_0/G_1 and $S+G_2/M$ cell fractions on a flow cytometer. Percentage of cells in G_0/G_1 and $S+G_2/M$ presort and post sort is shown for 4 independent experiments.

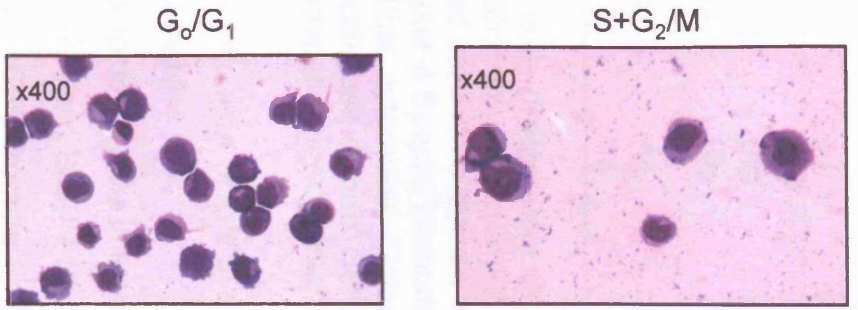


Figure 4.3 - Morphology of sorted G_0/G_1 and $S+G_2/M$ MPB $CD34^+$ cells after short term suspension culture. $CD34^+$ selected PBSCs were cultured in X-VIVO 10/1% HSA with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) for 48 hr and stained with Hst and Py Y. Cells were sorted on a flow cytometer for G_0/G_1 and $S+G_2/M$ cells. Sorted fractions were spun onto glass slides and stained with MGG. Representative cytospins of G_0/G_1 and $S+G_2/M$ cells are shown. Cells were viewed with the magnification indicated in each plate.

4.3.1.3 Effect of Hst/Pyronin Y staining and cell sorting on CFC numbers

In order to determine the effect of Hst/Pyronin Y staining and cell sorting on colony forming activity, I assessed the functional activity of freshly isolated haemopoietic progenitors and cytokine expanded cells (48hr cytokine stimulation) stained with Hst/Pyronin Y and sorted into G_0/G_1 and $S/G_2/M$ fractions in the methylcellulose culture. As shown in Table 4.2, G_0/G_1 and $S/G_2/M$ sorted cells generated comparable numbers of total colonies (Table 4.2).

Table 4.2 - Effect of Hst & Py staining and cell sorting on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Fresh	159	N/D	N/D	33	33
Pre-sort	160	N/D	N/D	70	68
Presort stained	106	N/D	N/D	N/D	45
G_0/G_1	54	40	46	34	N/D
$S+G_2M$	74	40	104	25	N/D

$CD34^+$ cells (fresh) were stimulated with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) in X-VIVO 10/1% HSA for 48hr (presort). Cultured cells were stained with Hst and Py Y (presort stained) as described in Chapter 4 Special Methods and sorted for G_0/G_1 and $S+G_2/M$ cell fractions on a flow cytometer. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 5 independent experiments.

4.3.1.4 Influence of cell cycle phase on short term BM homing of cytokine activated cells

In order to assess the influence of cell cycle phase on short term BM homing, sorted G_0G_1 and $S/G_2/M$ fractions (Figure 4.2B) of cultured $CD34^+$ cells were tested for CFC homing to the BM. The BM of animals transplanted with $CD34^+$ cells stimulated for 48 hr and sorted into G_0G_1 and $S/G_2/M$ fractions were recovered and single cell suspensions plated into semisolid culture. As shown in Figure 4.4, CFC from the G_0G_1 fraction transplanted animals showed a small increase (mean 0.5%, range 0.09-1.07%) in BM homing compared with their counterparts, CFC from the $S/G_2/M$ fraction (mean 0.3%, range 0.15-0.47%) however, this remained significantly lower than the

homing of un-stimulated cells (Chapter 3, Figure 3.10). The homing levels of CFC to the BM of unsorted (48hr cytokine stimulated) cells whether stained or unstained were 0.3% (mean, range 0.05-0.89%) for unstained and 0.2%, (mean, range 0.06-0.34%) for stained.

Parallel studies on the engraftment of these cell fractions confirmed that sorted G_0G_1 cells had no engraftment advantage compared with either unsorted cells or cells in the $S/G_2/M$ fraction (G_0G_1 cells [0.1% $CD45^+$], unsorted cells [0.2% $CD45^+$], and $S/G_2/M$ cells [0.1% $CD45^+$]).

4.3.2 Short term homing of cell cycle synchronised G_0/G_1, S and G_2/M cells

4.3.2.1 Aphidicolin induces cell cycle arrest of MPB $CD34^+$ cells

I cannot exclude the possibility that cell sorting might have an effect on homing capabilities of $CD34^+$ cells, or that sorted G_0G_1 cells did not display higher levels of homing because the critical cells important for homing and engraftment lay in the $S/G_2/M$ fraction. In order to address these issues, I used aphidicolin to induce cell cycle arrest at the G_1/S border prior to the first cell division (Huygen *et al*, 2002). $CD34^+$ cells were stimulated with SCF, Flt3L and TPO and aphidicolin was used to arrest cells at G_1/S border as detailed in the methods. As shown in Figure 4.5, freshly isolated $CD34^+$ cells only contain a small percentage of cells that are in cycle (4.6±1.4% in $S/G_2/M$), the majority of the cells reside in G_0G_1 phase of the cell cycle (Figure 4.1). After 40 hr of culture (ctl) the percentage of cells in cycle increases to 24.4±3.1%. However, treatment with aphidicolin (0) for 24hr induces cell cycle arrest and only 2.1± 0.9% of the cells are found in $S/G_2/M$ phase of the cell cycle (Figure 4.5). The aphidicolin treated cells were washed extensively and replated in fresh medium and cytokines. Cell cycle profiles of freshly isolated $CD34^+$ cells and after 40 hr of culture and treatment with aphidicolin from one representative experiment are shown in Figure 4.6. In this experiment, at the end of the 40 hour culture period, 95% of live cells treated with aphidicolin (Figure 4.6ii) were in G_0G_1 compared to 65% of control cells incubated in cytokines alone (Figure 4.6iii).

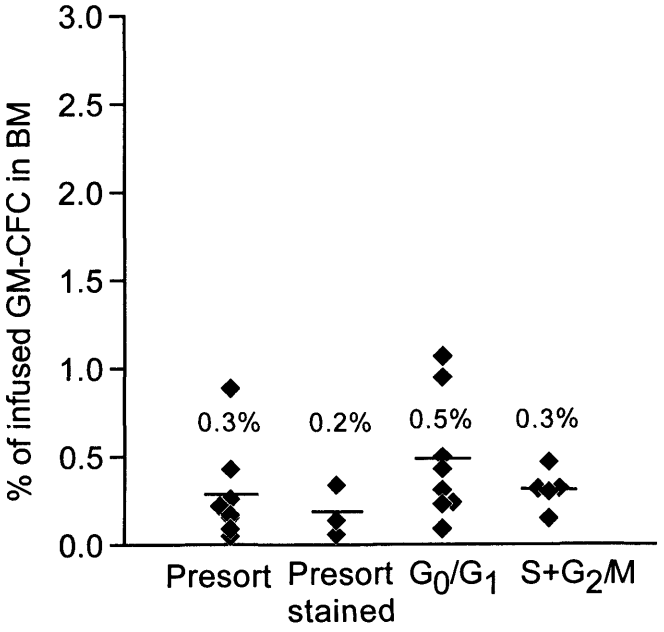


Figure 4.4 - Cell cycle fractionation of cytokine stimulated CD34$^+$ cells and the effect on homing. Short term BM homing of CD34$^+$ cells in G_0/G_1 or $S/G_2/M$. Sorted G_0/G_1 and $S/G_2/M$ fractions of cultured CD34$^+$ cells were infused (0.5-2.0x10^6/animal) into sub-lethally irradiated NOD/SCID animal and BM homing of progenitor cells was assessed as described in Chapter 2 General Methods. Cultured cells that were unstained (presort) or stained (presort stained) served as controls. The data are from 4 independent experiments, each data point represents 1 animal and numbers and bars indicate the means.

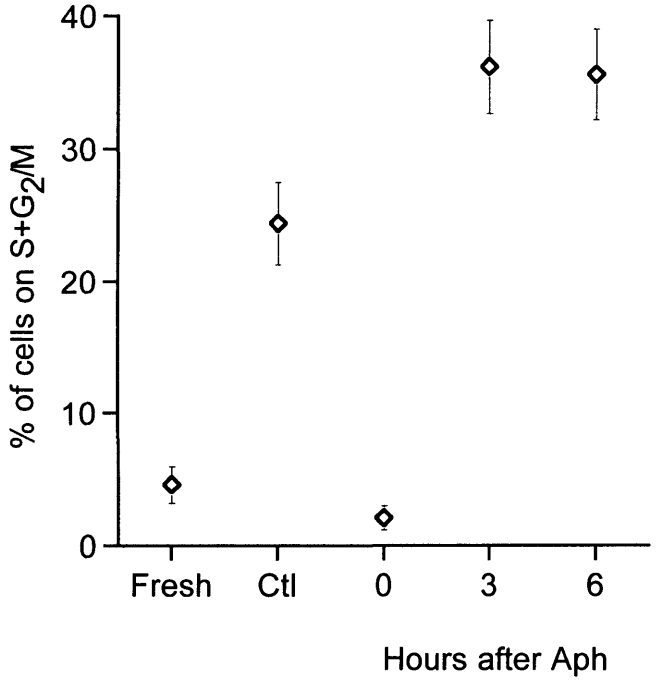


Figure 4.5 - Cell cycle synchronisation of cultured CD34$^+$ cells with aphidicolin. Freshly isolated CD34$^+$ cells reside in G_0/G_1 phase of the cell cycle (fresh). Cells were stimulated with SCF, Flt3L, TPO for 16hrs, after which they were reversibly blocked at the G_1/S transition by 24hr treatment with 2μg/ml aphidicolin (0) or kept in initial conditions (Ctl). Aphidicolin-treated cells were washed extensively and replated in fresh medium and cytokines to allow cell cycle progression. Cells entered S phase after 3hr (3) and reached G_2/M at 6 hr (6). Cell cycle status was determined by DNA staining with PI and percent cells in $S/G_2/M$ (mean ± SEM) of four independent experiments is shown.

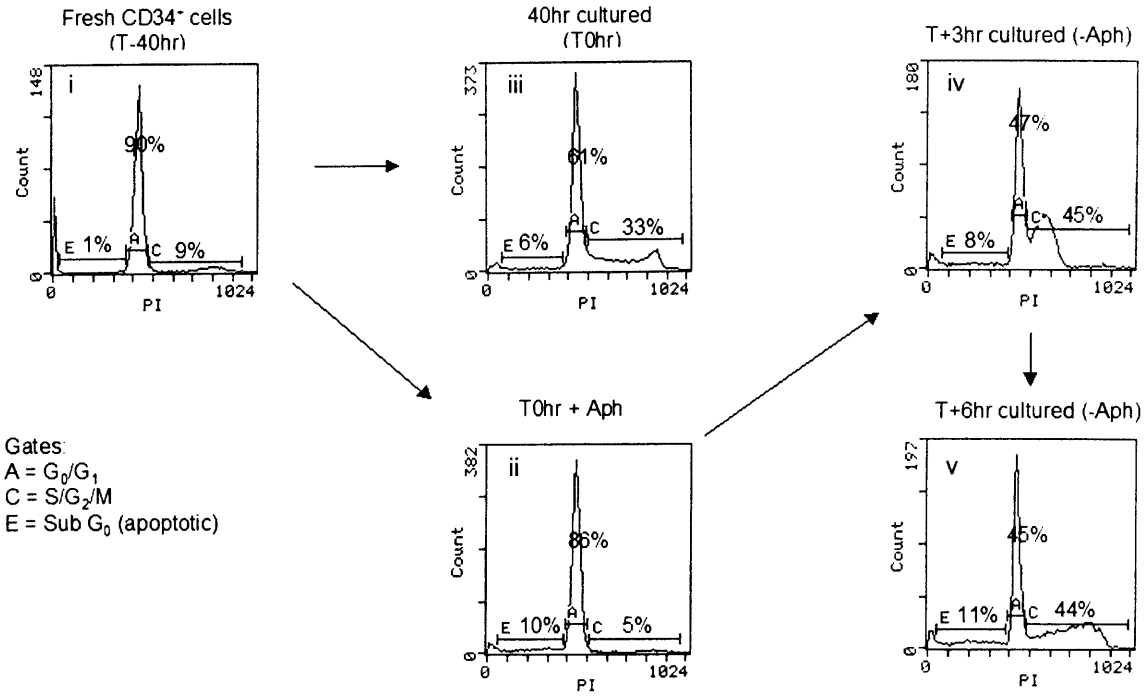


Figure 4.6 - Cell cycle synchronisation of cultured CD34$^+$ cells with aphidicolin. Freshly isolated CD34$^+$ cells reside in G_0/G_1 phase of the cell cycle (i). Cells were stimulated with SCF, Flt3L, TPO for 16hrs, after which they were reversibly blocked at the G_1/S transition by 24hr treatment with 2μg/ml aphidicolin (ii) or kept in initial conditions (iii). Aphidicolin-treated cells were washed extensively and replated in fresh medium and cytokines to allow cell cycle progression. Cells entered S phase after 3hr (iv) and reached G_2/M at 6 hr (v). Cell cycle status was determined by DNA staining with PI and percent cells in G_0/G_1 or $S/G_2/M$ of one representative experiment is shown.

After release from cell cycle block, cells moved into S (at 3 hours, Figure 4.6iv) and then G_2M (6 hours, Figure 4.6v) phases of the cell cycle in a synchronised manner. Gate E in Figure 4.6 indicates the proportion of apoptotic cells at each of these time points. Treatment with aphidicolin did not significantly alter the percentage of apoptotic cells (Figure 4.6 and Table 4.3). Cells were harvested at these time points of culture and infused into conditioned animals.

Table 4.3 - Cell cycle synchronisation of MPB $CD34^+$ cells with aphidicolin and effect on cell viability.

	% of cells in $SubG_0$		Mean
	Expt 1	Expt 2	
Fresh	1	1	1
Ctl	6	7	7
0	10	9	9
3	8	10	9
6	11	14	12

Freshly isolated $CD34^+$ cells (fresh) or cultured cells (ctl) were treated with aphidicolin (0) and then washed off and cell cycle progression continued for 3 and 6 hrs as described in Chapter 4 Special Methods. Cultured cells were stimulated with SCF, Flt3L and TPO at 100ng/ml in X-VIVO 10/ 1% HSA for 16hr, after which they were reversibly blocked at the G_1/S transition by 24hr treatment with 2μg/ml aphidicolin. Cell cycle status was determined by DNA staining with PI and percentage of cells in sub G_0 (apoptosis) in 2 independent experiments are shown.

4.3.2.2 Effect of aphidicolin cell cycle arrest on CFC numbers

I assessed the functional activity of freshly isolated haemopoietic progenitors and cells treated with aphidicolin in the methylcellulose colony assay *in vitro*. As shown in Table 4.4, aphidicolin cell cycle arrested cells generated comparable numbers of total colonies compared to control non-treated cells (ctl), n=2.

Table 4.4 - Effect of Aphidicolin cell cycle arrest on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	53	23
Ctl	88	43
0	74	32
3	90	24
6	85	30

Freshly isolated $CD34^+$ cells (fresh) or cultured cells (ctl) were treated with aphidicolin (0) and then washed and cell cycle progression continued for 3 and 6 hrs as described in Chapter 4 Special Methods. Cultured cells were stimulated with SCF, Flt3L and TPO at 100ng/ml in X-VIVO 10/ 1% HSA for 16hr, after which they were reversibly blocked at the G_1/S transition by 24hr treatment with 2μg/ml aphidicolin. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

4.3.2.3 Effect of cell cycle synchronisation on BM homing of cultured $CD34^+$ cells

Aliquots of cytokine activated $CD34^+$ cells, untreated and following treatment with aphidicolin as described above, were infused into conditioned NOD/SCID mice. As far as possible, equivalent cell numbers were infused for each of the conditions/time points tested. As seen in Figure 4.7, these different cell populations displayed very similar levels of BM homing (mean 0.5% of infused GM-CFC in BM), with the exception of cells at 3hr post aphidicolin block (45% in S+G_2/M phase) which achieved lower levels of homing (mean 0.2%, range 0.06-0.3%; p value 0.0094 compared with ctl, Mann-Whitney U test). Importantly, cells arrested at the G_1/S border displayed homing levels very similar to control cultured cells (mean 0.5%, range 0.43-0.54%) which had progressed unchecked through the cell cycle. Thus arresting cells at the G_1/S border did not restore the homing ability of cultured cells.

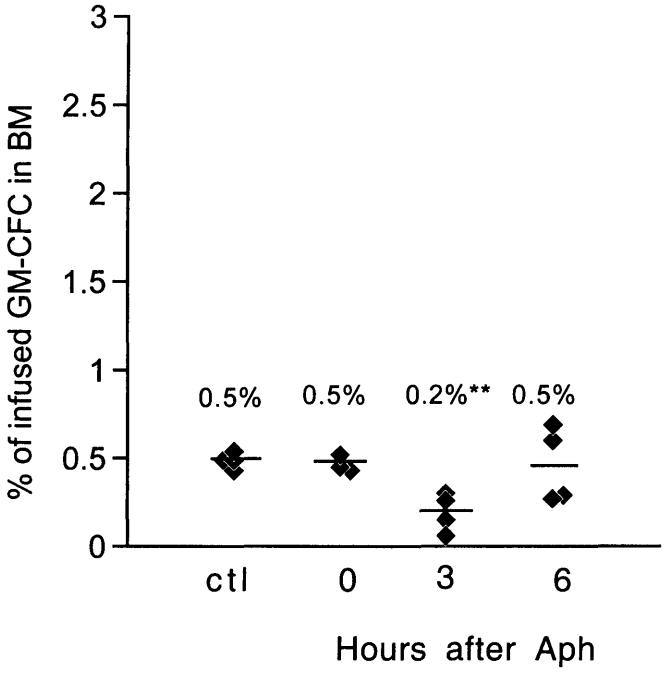


Figure 4.7 - Effect of cell cycle synchronisation on BM homing of cultured CD34$^+$ cells. Cell populations ii-v as indicated in Figure 4.6 were infused into NOD/SCID (1-2x10^{6}cells/animal). Homing of CFC to the BM was analysed at 24 hours. Data are given for individual animals in 2 independent experiments, mean number of colonies for each condition is indicated.
** $P<0.01$ compared with ctl; Mann-Whitney U test.

4.4 DISCUSSION

$CD34^+$ cells cultured with the 4 cytokine mixture entered cell cycle rapidly as shown by cell cycle analysis. With this cytokine combination, at 4hr of culture the majority (98%) of the cells were in G_O/G_1 phase of the cell cycle, but at 48 hr of culture this had fallen to only 72±2%. Cell cycle entry kinetics have been shown by (Giet *et al*, 2001) using MPB $CD34^+$ cells but cultured with the cytokine combination of SCF 100ng/ml, TPO 50ng/ml, and FltL 100ng/ml. Kinetics of MPB $CD34^+$ cell cycle entry have also been reported previously by (Yong *et al*, 1998) but using the cytokine combination of IL-3 12ng/ml, IL-6 10ng/ml and SCF 10ng/ml and in the presence of serum. I used the 4 cytokine mixture (SCF 100ng/ml, Flt3L 100ng/ml, IL-3 20ng/ml and IL-6 20ng/ml) and much higher concentration of SCF which resulted in a more rapid entry in to cell cycle, very similar in kinetics to the behaviour of UCB $CD34^+$ cells (20.2±1.2% of cells in $S+G_2/M$ phase of the cell cycle within 24hr) reported by (Yong *et al*, 1999). This difference in the kinetics of cell cycle entry of MPB $CD34^+$ cells may be due to the cytokine combination and the concentrations used.

MPB $CD34^+$ cells cultured with the 4 cytokine mixture for 48hr and stained with Hst and Py Y (DNA and RNA staining dyes) contained 31±4% cells in $S+G_2/M$. Similar results are obtained when cell cycle analysis is performed using PI (Figure 4.1). FACS sorting of proliferating $CD34^+$ cells stained with Hst and Py into G_0/G_1 and $S+G_2/M$ fractions yielded a purity of 98±0.3% and 83±4.7%, respectively. This level of purity is comparable to other reports in the literature for MPB $CD34^+$ cells (Giet *et al*, 2001) and UCB $CD34^+$ cells (Glimm *et al*, 2000). G_0/G_1 and $S+G_2/M$ sorted cells generated comparable numbers of GM-CFC, confirming previous reports (Glimm *et al*, 2000) using CB $CD34^+$ cells. Therefore cell staining and sorting does not adversely affect CFC formation and $CD34^+$ cells at different phases of the cell cycle contain equivalent numbers of CFCs.

Many workers have observed that the reduction in engraftment potential coincides with entry of cells into the active phases of the cell cycle (Habibian

et al, 1998;Gothot *et al*, 1997;Oostendorp *et al*, 2000) in both human and murine HSC. It has been suggested that homing ability and thus engraftment potential of cytokine activated $CD34^+$ cells alters during cell cycle transit, and is especially impaired during $S/G_2/M$ phase. The results I report here, however, do not support such an hypothesis. Although sorted G_0/G_1 cells showed slightly higher homing to the BM compared with sorted cells in $S/G_2/M$, this did not reach significance, and remained significantly lower than the levels seen with fresh cells. Importantly, sorted cells in different phases of the cell cycle (G_0/G_1 vs $S/G_2/M$) did not show differences in engraftment. My findings differ from those of Glimm and co-workers who used 5-day cultured cord blood $CD34^+$ cells and found that cells in S and G_2+M were ineffective in repopulation assays (Glimm *et al*, 2000) but engraftment after 6-8 weeks with both myeloid and lymphoid human cells was seen in mice transplanted with cells in G_0/G_1. The reasons for this difference are not clear but may relate to different culture conditions and to intrinsic differences between cord and adult $CD34^+$ cells. The effect of cytokine culture may be different for ontogenically earlier cells such as cord and foetal HSC (Wilpshaar *et al*, 2000;Wilpshaar *et al*, 2002). Moreover, in this as in other studies, engraftment of cytokine activated cells was not compared with the levels achieved for fresh cells. My findings on *in vivo* homing of cytokine stimulated $CD34^+$ cells also contrast with observations that the *in vitro* adhesive function of these cells alters with cell cycle. Yong *et al.,* (2002b) and Huygen *et al.,* (2002) have found that cultured $CD34^+$ cells exhibit increased migration and reduced adhesion whilst in G_0/G_1 phase of the cell cycle compared to cells in $S/G_2/M$ (Yong *et al*, 2002b;Huygen *et al*, 2002). It is clear however that extrapolation of these *in vitro* observations to the *in vivo* mechanisms whereby circulating $CD34^+$ cells home to the BM must be made with caution.

In the second approach I used aphidicolin (a tetracyclic diterpene with anti-mitotic properties) to synchronise the cells at the G_1/S transition. Huygen *et al.,* (2002) have previously demonstrated synchronisation of UCB $CD34^+$ cells using aphidicolin and monitored changes in adhesion and motility at various stages during a single cell cycle transit (Huygen *et al*, 2002). Using similar culture conditions, I have demonstrated that MPB $CD34^+$ cells

stimulated with SCF, Flt3L and TPO for 16 hr can also be arrested by treatment with aphidicolin for 24hr. This block at the G_1/S border and subsequent release by washing away the aphidicolin allowed synchronisation of cells as they progress into the S+G_2/M phases of the cell cycle. In my experiments aphidicolin had no significant influence on CFC formation as previously reported by (Huygen *et al*, 2002) on UCB $CD34^+$ cells. I also demonstrated that aphidicolin treatment has no significant influence on cell viability because there is not a significant difference in sub-G_0 (apoptotic) population of cells treated with or without aphidicolin. However, cells induced to accumulate at the G_1/S border by treatment with aphidicolin did not show increased homing to the BM, compared with control cultured cells. The lower homing levels of cells in S-phase is interesting but does not explain the impaired BM homing of cultured cells.

Therefore both the above strategies have shown no significant difference in homing levels to the BM of G_0/G_1 vs $S/G_2/M$ cytokine stimulated MPB $CD34^+$ cells in the NOD/SCID mice. These results for adult MPB $CD34^+$ cells contrasts with previous reports on UCB cells albeit using different culture conditions. My observations suggest that other factors apart from cell cycle status are likely to influence the homing and engraftment capabilities of adult HSPC.

In summary, I have demonstrated that in adult $CD34^+$ cells cytokine induced loss of BM homing to the BM is a phenomenon that occurs rapidly and does not appear to relate to cell cycle progression.

Chapter 5: Strategies for improving homing of cytokine stimulated MPB $CD34^+$ cells

5.1 INTRODUCTION

Cytokines and ex vivo expansion

Stimulatory cytokines have been utilised for *ex vivo* expansion of HSPC to aid stem cell transplantation or to optimise retroviral gene transfer. Additionally, many investigators have explored the possibility of accelerating engraftment by transplanting haemopoietic cells that have been expanded and partially differentiated *in vitro* by culturing in the presence of potent combinations of haemopoietic growth factors (Srour *et al*, 1999). Efforts to attain the maximal benefit of *ex vivo* expanded haemopoietic cells are however, currently hampered by deleterious changes, acquired in culture, in the engraftment ability of these cells, as described in previous sections.

It is thought that the loss of long term engraftment potential during culture is due to deleterious changes in the ability of these cells to home to the BM. The data presented in Chapter 3 confirm that adult $CD34^+$ cells undergo a change in their *in vivo* homing behaviour following a period of culture *in vitro*. This change resulted in a significant decrease in the homing of cells to the BM. In Chapter 4, I tested the hypothesis that these changes in homing behaviour are cell cycle dependent, but found no difference between cells in different phases of the cell cycle. Furthermore, blocking G_1/S transition in cytokine-stimulated cells did not restore the loss of BM homing ability.

impaired BM homing

Therefore, perhaps nothing to do with cell cycle but the result of cytokine-stimulation. Indeed, HSPC from foetal and cord blood are able to engraft despite the fact they contain a significant proportion of cells in active phases of the cell cycle (Wilpshaar *et al*, 2000;Wilpshaar *et al*, 2002). Therefore the altered homing ability may be the result of cytokine activation, and manipulating the particular cytokine combination, or conditions of culture, may prevent the loss of BM homing.

Several combinations of cytokines known to act on primitive HSCs have been employed *in vitro* in an attempt to produce culture conditions suitable for HSC expansion (reviewed in (Moore, 2002)). The ligand for c-kit (SCF) and Flt3

Ligand known to transduce signals crucial for HSC proliferation, and the c-mpl ligand, TPO shown to stimulate primitive HSC expansion, have all been regarded as key factors for triggering self–renewal (Williams *et al*, 1992;Rosnet *et al*, 1993;Matthews *et al*, 1991;Small *et al*, 1994;Young *et al*, 1996;Yoshida *et al*, 1997;Sui *et al*, 1995;Knobel *et al*, 1994;Conneally *et al*, 1998;Conneally *et al*, 1997;Bhatia *et al*, 1997;Piacibello *et al*, 1997;Ballen *et al*, 2000;Gammaitoni *et al*, 2003;Verfaillie, 2002b). Studies by (Conneally *et al*, 1997) and by others (Dao *et al*, 1997;Demaison *et al*, 2000), have shown that inclusion of Flt3L with the combination of IL-3, IL-6 and SCF augments the survival of the stem cells while they are in culture. On BM stem cells, only the so-called early acting growth factors such as TPO, SCF, Flt3L and IL-3 are able to promote a net expansion of LTCICs, whereas the amplification of CFCs requires the presence of IL-6 and /or G-CSF (Petzer *et al*, 1996). Identification of these differential effects of cytokines on PBSC is therefore important in order to develop improved clinical expansion protocols aimed at both amplification of committed progenitors to shorten the neutropenic period and maintenance of the primitive stem cells with long term engraftment ability (Herrera *et al*, 2001). As indicated in the above studies the specific cytokine combination used in *ex vivo* expansion may play a major role in maintaining the repopulating ability of these cells, perhaps by influencing their homing ability.

Recently, much interest has focussed on modifying culture conditions so as to restore the engraftment capability of pre-activated cells. Interleukin-3 (IL-3) has been implicated in the loss of long term reconstituting activity of cultured HSC (Yonemura *et al*, 1996), while cytokines which selectively stimulate primitive HSC, such as TPO, SCF and FL, have been reported to preserve long term repopulating cells (Dao *et al*, 1997;Luens *et al*, 1998;Verfaillie, 2002b). It is difficult to make direct comparison between such studies, which vary in terms of cell source, the use of serum free media, and the *in vivo* model (murine, or NOD/SCID, or SCID-hu bone) and not all take into account the degree of cell expansion after culture. Nevertheless, in HSC stimulated to divide following exposure to TPO/SCF/FL *in vitro*, NOD/SCID repopulating activity is maintained and is demonstrated by cells in the post-mitotic

compartment (Danet *et al*, 2001;Herrera *et al*, 2001). If actively cycling HSPC have an engraftment defect, then modulating culture conditions to induce quiescence at time of infusion might restore the repopulating potential. Exposure to TGF-β increased the proportion of HSPC in G_0G_1 phase of the cell cycle, but did not aid engraftment in either a murine model or xenogeneic model (Wiesmann *et al*, 2000;Glimm *et al*, 2000). In a recent study, preactivated primate $CD34^+$ cells were "rested" for 2 days in SCF and retronectin resulting in an increase in the proportion of cells in G_0G_1 and in superior engraftment compared with control 'non-rested' cells (Takatoku *et al*, 2001).

SDF-1 has recently been reported to enhance survival, while inhibiting cycling of HSPC (Cashman *et al*, 2002;Glimm *et al*, 2002). Cashman *et al.,* (2002) provided evidence that SDF-1 can function as an inhibitor of human LT-CIC cycling and inhibited the cycling activity of HPP-CFC, but not the more mature LPP-CFC (Cashman *et al*, 2002). Incubation of proliferating cord blood $CD34^+$ cells with SDF and TPO produced an increase in CRU frequency, although without any changes in the cell cycle profile of the cells (Glimm *et al*, 2002). In combination with other cytokines, SDF-1 was actually found to promote the proliferation of human $CD34^+$ cells purified from normal adult PB although when SDF-1 was added alone, only survival was supported, not mitogenesis (Lataillade *et al*, 2000).

Fas expression and $CD34^+$ cell homing

My finding in Chapter 4 and studies by Young *et al.,* (2001) and Liu *et al.,* (2003) have shown that Fas receptor expression is upregulated on both MPB and UCB $CD34^+$ cells during culture (Young *et al*, 2001;Liu *et al*, 2003). This may make these cells more susceptible to apoptosis *in vivo* when encountering Fas Ligand e.g. on surface of endothelium (Josefsen *et al*, 1999). For example, Fas Ligand within the mouse vasculature could induce aggregation of Fas receptor on cells post injection and trigger apoptosis, this could then contribute to the observed homing and engraftment defect of cultured cells. Young *et al.,* (2001) observed that caspase inhibitors of Fas-induced apoptosis significantly improved engraftment (2-3 fold) in a murine

xenogeneic transplantation model (SCID-hu mouse) (Young *et al*, 2001). These authors suggested that cells with up-regulated Fas R expression were being protected by caspase inhibitor from apoptosis triggered by *in vivo* Fas L during homing and engraftment (Young *et al*, 2001).

A recent study using cord blood $CD34^+$ cells found that the reduced homing levels of cultured CFC could be restored by incubating cells prior to transplantation with Fas/CD95 blocking mAb ZB4 (Liu *et al*, 2003). This is another approach used to protect cells from undergoing apoptosis by *in vivo* Fas L during homing and engraftment. This approach was used in this Chapter to determine whether increased Fas/CD95 levels caused the homing defect of cultured $CD34^+$ cells from adult MPB.

Chapter Aims

I tested several *ex vivo* manoeuvres including several different culture conditions which have been reported to restore the engraftment of preactivated cells, to investigate if such strategies were able to modulate the homing behaviour of adult $CD34^+$ cells.

5.2 Special Methods

5.2.1 Fas/CD95 blockade

$CD34^+$ cells were stimulated in tissue culture flasks (Nalge Nunc) at a density of $0.5x10^6$/ml with SCF, Flt3L (100ng/ml) and IL-3, IL-6 (20ng/ml) in X-VIVO 10/1%HSA at 37°C and 5% CO_2. In experiments using a blocking mAb, 48hr-cultured cells were pre-incubated for 30 minutes on ice with anti-Fas mAb (clone ZB4; mouse IgG_1;MBL International, Watertown, MA) at 2μg/ml or 5μg/ml. Cells were not washed but injected in the presence of antibody in maximum volume of 500μl in HBSS.

In a second set of experiments, human recombinant soluble Fas Ligand at 10, 50 and 100ng/ml (sFasL, Alexis Corporation Ltd) was included in the culture medium (48-72 hours). Before transplantation, cells were washed with

10ml HBSS and resuspended in HBSS supplemented with sFasL for injection into NOD/SCID mice.

In order to confirm that anti-Fas mAb (ZB4) was functional, Jurkat T cells were preincubated with ZB4 and apoptosis triggered with FasL Plus recombinant protein (Oncogene). Jurkat T cells (purchased from ATCC) were grown in RPMI supplemented with 10% FCS and maintained at a cell density of $2x10^5$/ml at 37°C and 5% CO_2. Jurkat T cells were seeded into a 24 well plate at $2x10^5$/ml in RPMI/10% FCS alone or with FasL plus (0.1, 1, and 5ng/ml) with or without preincubation with ZB4 at 5μg/ml for 30 minutes at 37°C. The proportion of apoptotic cells was determined after 6 and 24 hr.

5.2.2 Apoptosis assay

For apoptosis evaluation, cells were pelleted at 1600rpm for 10 minutes and then washed once in Annexin V binding buffer (140mM NaCl, 10mM HEPES, pH 7.4 and 5mM $CaCl_2$) before incubation for 5 minutes at room temperature with 1μl Annexin V-FLUOS (Roche) in 100μl annexin V binding buffer per sample. Specific fluorescence was then determined using an EPICS Elite flow cytometer (Beckman-Coulter).

5.2.3 Retronectin coated plates

Retronectin treated plates were prepared by adding RetroNectin at 10μg/ml (TaKaRa Biomedicals T100A) to each well of a 6 well tissue culture plate for 2 hours at room temperature, followed by blocking with 2% BSA in PBS w/o Ca^{++} Mg^{++} for 30 minutes at room temperature and finally three washes in PBS.

5.3 RESULTS

5.3.1 Role of culture conditions

5.3.1.1 Effect of IL-3 on the homing of cytokine activated CD34$^+$ cells

Omitting IL-3 from my cultures had no significant effect on the homing behaviour of cytokine activated cells (Figure 5.1). Cells cultured in the presence or absence of IL-3 produced similar levels of GM-CFC homing to the BM (0.4% for both, Figure 5.1). Cells cultured with or without IL-3 generated comparable numbers of total colonies as fresh cells, n=2 (Table 5.1).

Table 5.1 - Effect of IL- 3 on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	106	108
-IL-3	94	106
+IL-3	N/D	102

Freshly isolated CD34$^+$ cells (fresh) were stimulated in SCF, Flt3L, IL-3, IL-6 (+IL-3) or SCF, Flt3L, TPO (-IL-3) in X-VIVO 10/1% HSA for 48hr as described in Chapter 2 General Methods. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

5.3.1.2 Effect of SDF-1 and of retronectin on the homing of cytokine activated CD34$^+$ cells

Alterations in homing and engraftment may be dependent upon the particular culture conditions employed. Recent reports suggest that, in addition to its chemotactic properties, SDF-1 may also be important in the survival and cell cycle behaviour of CD34$^+$ cells (Cashman *et al*, 2002;Glimm *et al*, 2002). Exposure of 4-day activated CD34$^+$ cells to SDF-1 for a further 2 days increased the proportion of cells in G_0G_1 (from 72% to 85%). These cells generated comparable CFC numbers as compared with control cells cultured with and without SDF-1, and fresh non-cultured cells (Table 5.2). Despite the increase in G_0/G_1 fraction, SDF-1 exposed cells displayed similar homing levels to the BM to that seen with control CD34$^+$ cells cultured in the absence of SDF-1 (Figure 5.2).

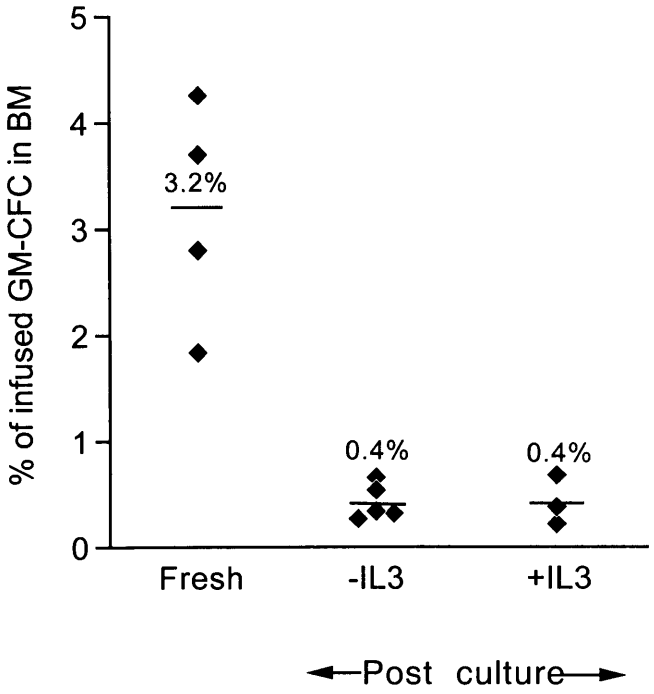


Figure 5.1 - Effect of varying culture conditions on homing of $CD34^+$ cells in NOD/SCID mice. Freshly isolated $CD34^+$ cells (fresh) were compared to cells stimulated in the presence or absence of IL-3 as indicated and 24hr BM homing of CFC determined. The data are from 2 independent experiments, each data point represents 1 animal, and the mean values are indicated.

Table 5.2 - Effect of SDF-1 and retronectin on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	117	60
Ctl	134	71
+2d	94	29
+2d SCF, TPO	92	N/D
+2d SCF, TPO, SDF1	87	44
Ctl Rn	N/D	77
+2d Rn	N/D	56

$CD34^+$ cells were cultured for 4 days in SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) on retronectin (ctl Rn) or without (ctl). Some cells were washed and replated for a further 2 days in the same cytokines (+2d), or on retronectin (+2d Rn) or with SDF1 (+2d SCF, TPO, SDF1) as indicated. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

Finally, culture on retronectin has been reported to result in superior engraftment potential (Takatoku *et al*, 2001). We included retronectin in our standard cytokine culture, or took 4-day cultured $CD34^+$ cells, washed and re-plated them on retronectin in the presence of SCF and tested for BM homing ability in conditioned animals. Cells cultured under these different conditions generated comparable numbers of CFCs (Table 5.2). We did not find that either of these culture methods using retronectin was able to restore the homing levels of cytokine activated $CD34^+$ cells (Figure 5.2). As shown in Figure 5.2, cells cultured with or without retronectin showed very similar mean levels of homing to the BM (0.17, 0.22, and 0.25%).

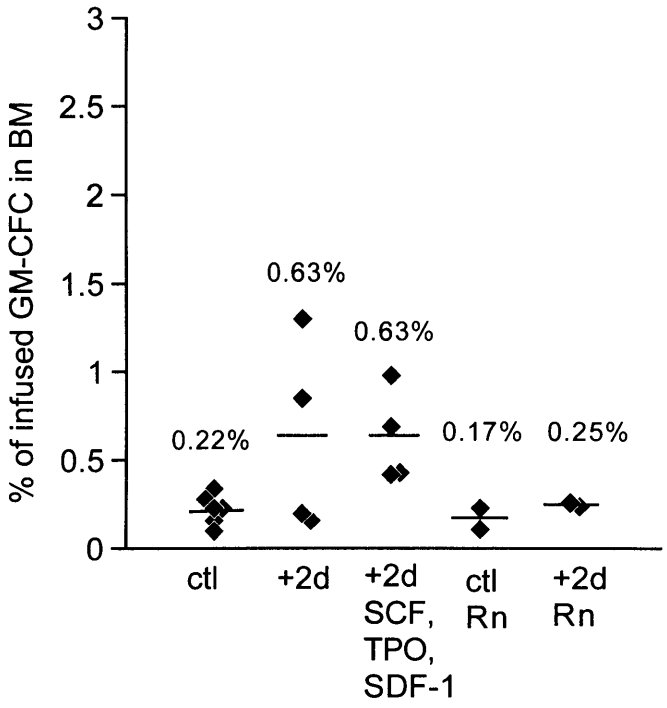


Figure 5.2 - Effect of varying culture conditions on homing of CD34$^+$ cells in NOD/SCID mice. CD34$^+$ cells were cultured for 4 days in SCF, Flt3L, IL-3 and IL-6 on retronectin (ctl Rn) or without (ctl). Some cells were washed and re-plated for a further 2 days in the same cytokines (+2d), or on retronectin (+2d Rn) or with SDF (+2d SCF, TPO, SDF-1) as indicated. At the end of the culture period, cells were washed and injected into conditioned animals ($2x10^6$ cells/animal) and 24hr BM homing of CFC determined. The data are from 2 independent experiments, each data point represents 1 animal, and the mean values are indicated.

5.3.2 Role of Fas-ligand:

The findings in Chapter 4 suggest that cell cycle progression could not explain the reduced BM homing behaviour of cytokine activated $CD34^+$ cells. Among the surface receptors which are altered by cytokine exposure of $CD34^+$ cells is Fas. As seen in Chapter 3, Figure 3.2, Fas expression on $CD34^+$ cells increased from 6±1% to 65±9% after 2 days of culture, $P<0.01$. Ligation of surface Fas molecules with endogenous Fas-ligand *in vivo* may lead to premature destruction of infused cells. I used 2 different approaches to attempt to block Fas engagement *in vivo*.

5.3.2.1 CD95/Fas blocking mAb (ZB4) inhibits the apoptosis of Jurkat T cells

In the initial experiment, I confirmed that ZB4 was able to inhibit the apoptosis of Jurkat T cells triggered by FasL Plus. Jurkat T cells were cultured in RPMI/10% FCS with either FasL Plus or pre-incubated with ZB4 (5μg/ml) for 30 minutes and then incubated with FasL Plus, as described in Chapter 5 Special Methods. The percentage of annexin V positive cells was determined at both 6 and 24hr of culture by flow cytometry. Control Jurkat T cells cultured in RMPI/10% FCS contained 19% annexin v positive cells after 6 hrs. Culture of cells with FasL Plus at 0.1 and 1ng/ml resulted in 17 and 14% Annexin v positive cells, respectively. In contrast culture with FasL Plus at a concentration of 5ng/ml reduced cell viability and 43% of Jurkat T cells were annexin v positive. Pre-incubation with ZB4 prior to culture with FasL Plus (5ng/ml) blocked this increase in apoptosis (Table 5.3). This confirmed that ZB4 mAb was capable of blocking apoptosis of Jurkat T cells. Annexin V staining of Jurkat T cells at 24hr of culture showed a similar trend (Table 5.3), with a decrease in cell viability following exposure to FasL Plus at 5ng/ml, (37% of cells annexin v positive). Pre-treatment with ZB4 resulted in blockade of apoptosis (reduced to 13%, Table 5.3).

Table 5.3 - Blocking apoptosis in Jurkat T cells using ZB4.

	% annexin v positive cells	
	6hr	24hr
Control	19	5
FasL 0.1ng/ml	17	11
FasL 1ng/ml	14	20
FasL 5ng/ml	43	37
ZB4 + FasL 0.1ng/ml	20	13
ZB4 + FasL 1ng/ml	20	12
ZB4 + FasL 5ng/ml	17	13

Jurkat T cells were cultured in RPMI/10% FCS (control) with either FasL Plus® at 0.1, 1, 5ng/ml or pre-incubated with ZB4 (5ug/ml) for 30 minutes and then incubated with FasL Plus® (0.1, 1, 5ng/ml) as detailed in Chapter 5 Special Methods. Percentage annexin v positive cells was determined at 6 and 24hr of culture by flow cytometry. Data are shown from a representative experiment.

5.3.2.2 Effect of Fas/CD95 blocking mAb (ZB4) on CFC numbers

In order to exclude any negative impact of ZB4 incubation on CFC activity. I assessed the functional activity of freshly isolated haemopoietic progenitors and $CD34^+$ cells cultured for 48hr in the 4 cytokine mix in the presence or absence of ZB4 in the methylcellulose culture. As shown in Table 5.4, cells treated with ZB4 at 2 and 5μg/ml generated comparable numbers of total colonies compared with control cells cultured for 48 hr in the absence of ZB4, n=2.

Table 5.4 - Effect of ZB4 on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	82	76
Cultured	89	181
ZB4 2μg/ml	94	101
ZB4 5μg/ml	103	101

Freshly isolated $CD34^+$ cells (fresh) were stimulated with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) in X-VIVO 10/1% HSA for 48hr (0) and then incubated with ZB4 at 2 or 5μg/ml for 30 minutes on ice. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

5.3.2.3 Blocking Fas ligation with the blocking anti-Fas mAb (ZB4)

In the first approach to attempt to block Fas engagement *in vivo*, I pre-incubated cytokine stimulated $CD34^+$ cells with the blocking anti-Fas mAb, ZB4 prior to infusion. Freshly isolated haemopoietic progenitors and $CD34^+$ cells cultured for 48hr in the 4 cytokine mixture in the presence or absence of ZB4 were infused by the tail vein route into NOD/SCID mice. Mononuclear cells recovered from murine BM 24 hr post transplantation were plated into semisolid culture for enumeration of human CFC, as described in Chapter 2 General Methods. Aliquots of pre infusion $CD34^+$ cells were set up in parallel methylcellulose culture and used as reference in order to quantify homing efficiency of CFC. As shown in Figure 5.3, this manoeuvre was not successful in rescuing the impaired homing of 48-hour cultured $CD34^+$ cells. Cytokine stimulation resulted in a marked reduction in the homing of CFC to the BM when compared to fresh cells (fresh GM-CFC mean 3.6% vs 0.4% for cultured cells), Figure 5.3, (Chapter 3, Figure 3.8a). Cytokine stimulated cells pre-incubated with blocking mAb ZB4 showed lower levels of CFC homing to the BM (0.02% of infused GM-CFC in BM). Homing of fresh and post cultured cells treated with or without ZB4 to the spleen was also analysed, as the spleen is a haemopoietic site in the mice. Cytokine activated $CD34^+$ cells showed a reduction in homing to the spleen (Table 5.5), as seen previously in Chapter 3, Figure 3.8B. As shown in Table 5.5, fresh $CD34^+$ transplanted mice formed 67, 52 and 98, 86 GM-CFC colonies per 10^6 murine spleen cells for experiment 1 and experiment 2, whereas cultured $CD34^+$ transplanted mice formed less colonies 15, 96 and 38, 24 for experiment 1 and experiment 2, respectively. In contrast ZB4 treated $CD34^+$ transplanted mice formed lower colonies 5, 9, 2 and 0, 5, 4 GM-CFC colonies per 10^6 murine spleen cells for experiment 1 and experiment 2, respectively.

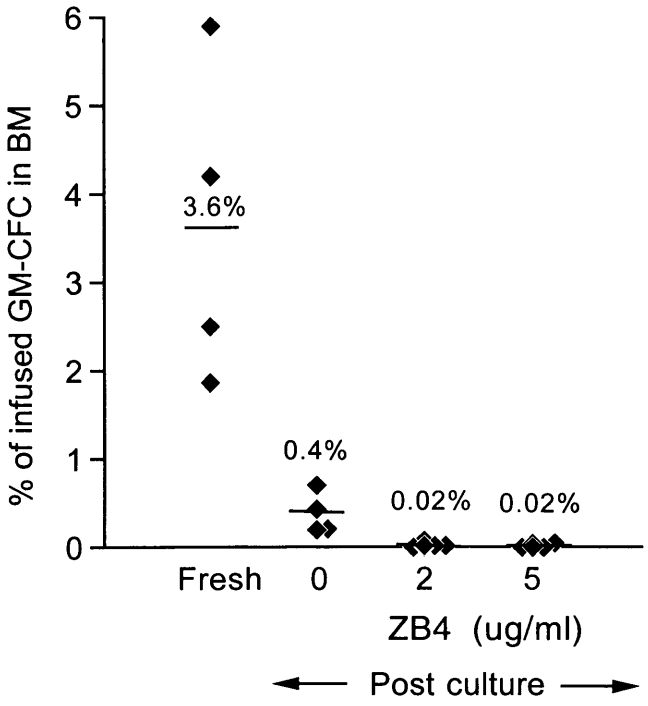


Figure 5.3 - Blocking Fas ligation and effect on homing. Cultured $CD34^+$ cells were pre-treated with ZB4 (2 and 5μg/ml) for 30 minutes before transplantation into animals ($2x10^6$ cells/animal). Short term BM homing was assayed 24 hours post infusion. The homing of fresh cells is shown for comparison. The data are from 2 independent experiments, each data point represents 1 animal, and the mean values are indicated.

Table 5.5 - Homing of MPB $CD34^+$ cells to the spleen: effect of ZB4

	No. of GM-CFC per $1x10^6$ cells	
	Expt 1	Expt 2
Fresh	67, 52	98, 86
Cultured	15, 96	38, 24,
ZB4 2μg/ml	0, 0, 21	0, 0, 0
ZB4 5μg/ml	5, 9, 2	0, 5, 4

Freshly isolated $CD34^+$ cells (fresh) were stimulated with SCF (100ng/ml), Flt3L (100ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) in X-VIVO 10/1% HSA for 48hr (Cultured) and then incubated with ZB4 at 2 or 5μg/ml for 30 minutes on ice before transplantation into animals ($2x10^6$ cells/animal). Homing of cells to the spleen at 24hr post transplantation is shown. Each data point represents one animal. Data are presented as GM-CFC per million spleen cells from 2 independent experiments.

5.3.2.4 Blocking Fas ligation with sFasL

In the second approach to attempt to block Fas engagement *in vivo*, I treated cytokine stimulated $CD34^+$ cells with sFasL, which has been suggested to have a protective role by competing against membrane-bound Fas-ligand (Josefsen *et al*, 1999). Freshly isolated $CD34^+$ cells were cultured for 4 days in the 4 cytokine mixture and treated with sFasL before transplantation into mice. Cells cultured under these conditions generated comparable numbers of total colonies as control cells (Table 5.6). As above mononuclear cells recovered from murine BM 24 hr post transplantation were plated into semisolid culture for enumeration of human CFC, as described in Chapter 2 General Methods. Aliquots of pre infusion $CD34^+$ cells were set up in parallel methylcellulose culture and used as reference in order to quantify homing efficiency of CFC. Figure 5.4 shows that this manoeuvre was not successful in rescuing the impaired homing of 48-hour cultured $CD34^+$ cells. $CD34^+$ cells cultured for 4 days resulted in 0.38% of GM-CFC homing to the BM, in comparison the same cells treated with sFasL at 10, 50 and 100ng/ml displayed similar levels of homing to the BM (0.13, 0.16 and 0.11%, respectively).

Table 5.6 - Effect of sFasL on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells	
	Expt 1	Expt 2
Fresh	53	59
Cultured	75	130
sFasL 10ng/ml	70	128
sFasL 50ng/ml	78	88
sFasL 100ng/ml	59	134

Freshly isolated $CD34^+$ cells (fresh) or cultured cells (0) were treated with sFasL at 10, 50, 100ng/ml as described in Chapter 5 Special Methods. Colony forming activity of cells was assessed in methylcelluose culture. Data are expressed per 1000 cells plated and given for 2 independent experiments.

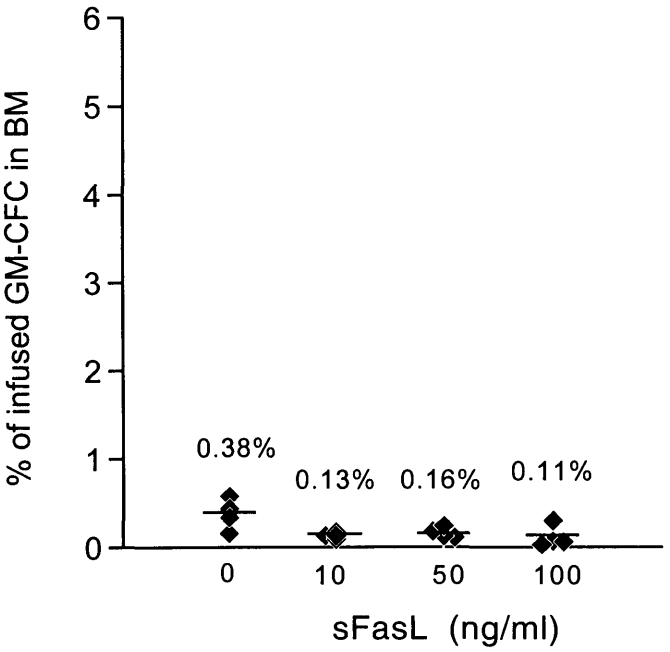


Figure 5.4 - Blocking Fas ligation and effect on homing. Cultured $CD34^+$ cells were treated with sFasL at 10, 50, and 100ng/ml before transplantation into animals ($2x10^6$ cells/animal). Short term BM homing was assayed 24 hours post infusion. The data are from 2 independent experiments, each data point represents 1 animal, and the mean values are indicated.

5.4 DISCUSSION

In this chapter, I evaluated several different strategies to augment the BM homing of cultured adult $CD34^+$ cells. Alterations in homing and engraftment may be dependent upon the particular culture conditions employed. Studies by (Conneally *et al*, 1997) and by others (Dao *et al*, 1997;Demaison *et al*, 2000), have shown that inclusion of Flt3L with the combination of IL-3, IL-6 and SCF augments the survival of the stem cells while they are in culture. In contrast other studies have shown that inclusion of IL-3 in stimulation cultures may be detrimental to engraftment potential of expanded cells (Herrera *et al*, 2001). Interleukin-3 (IL-3) has been implicated in the loss of long term reconstituting activity of cultured HSC (Yonemura *et al*, 1996), while cytokines which selectively stimulate primitive HSC, such as TPO, SCF and FL, have been reported to preserve long term repopulating cells (Dao *et al*, 1997;Luens *et al*, 1998). Such effects may be mediated at the level of homing to haemopoietic tissue. Thus, I addressed the effect of manipulating the *ex vivo* culture conditions on the homing of cytokine activated $CD34^+$ cells.

In order to determine if IL-3 in the cytokine cocktail was able to modulate the homing behaviour of adult $CD34^+$ cells, cells were stimulated with Flt3L, SCF, IL-6 with or without IL-3, and tested for homing in the NOD/SCID. Ommiting IL-3 from my cultures had no significant effect on the homing behaviour of cytokine activated cells. This is in contrast to the finding of (Herrera *et al*, 2001) who observed that groups of mice transplanted with cells cultured with TPO+SCF+FL showed higher levels of human engraftment when compared with mice injected with the similar cell doses of $CD34^+$ cells cultured with TPO+SCF+FL+IL-3+IL-6. In the latter group, no evidence of human engraftment was found at any of the doses tested (Herrera *et al*, 2001). They conclude that when IL-3 and IL-6 were added to the culture there is a loss of ability to engraft NOD/SCID mice. On the other hand, other groups have found that IL-3 and IL-6 do not compromise the engraftment potential of primitive stem cells (Conneally *et al*, 1997;Piacibello *et al*, 1999).

As reviewed in Chapter 4, many studies suggest that actively cycling HSPCs have an engraftment defect when compared with quiescent cells in G_0 phase of the cell cycle (Gothot *et al*, 1998). In the murine model, there is some evidence that this defect may be reversible, if cells are allowed to come back out of active cycle before transplantation (Habibian *et al*, 1998). In a recent study, preactivated primate $CD34^+$ cells were "rested" for 2 days in SCF and retronectin resulting in an increase in the proportion of cells in G_0G_1 and in superior engraftment compared with control 'non-rested' cells (Takatoku *et al*, 2001). Similarly SDF-1 has recently been reported to enhance survival, while inhibiting cycling of HSPC (Cashman *et al*, 2002;Glimm *et al*, 2002). Incubation of proliferating cord blood $CD34^+$ cells with SDF and TPO produced an increase in CRU frequency, although without any changes in the cell cycle profile of the cells (Glimm *et al*, 2002).

In order to determine if the above mentioned approaches were able to improve homing of MPB $CD34^+$ cells, I treated freshly isolated $CD34^+$ cells with SDF-1 and retronectin as described in Chapter 2 General Methods. I firstly demonstrate that cells cultured with SDF-1 and on retronectin generated comparable numbers of GM-CFCs and therefore these culture conditions had no significant effect on the colony formation of the $CD34^+$ cells. This has been shown by (Glimm *et al*, 2002) using UCB cells and found no significant difference in fold increase in CFC between cells cultured in SF+TPO vs SF+TPO+SDF-1. The role of SDF-1 in inhibiting the cycling of primitive HSPC (UCB and adult BM) *in vitro* has been shown previously by (Cashman *et al*, 2002;Glimm *et al*, 2002). I demonstrated that exposure of 4-day activated MPB $CD34^+$ cells to SDF-1 for a further 2 days increased the proportion of cells in G_0/G_1 from 72% to 85% but without any effect on BM homing ability. My findings differ from those of (Plett *et al*, 2002) who used 2-3 day cultured MPB $CD34^+$ cells and found that in 3 of 4 experimental groups, recovery of SDF-1α pretreated (100ng/ml) MPB $CD34^+$ cells in NOD/SCID BM 26-40 hours post injection was increased compared to cells not exposed to SDF-1α i.e. cultured in IMDM/10% FCS only (fold increase 8.6, 14.7, 0.7, 1.3; n=1-2 mice/group). They also demonstrated that SDF-1α

pretreatment of MPB $CD34^+$ cells resulted in 40-50% higher chimerism in NOD/SCID mice while pretreatment of BM $CD34^+$ cells with SDF-1α did not affect the engraftment potential. The reason for this difference is not clear but may relate to the use of IMDM and FBS in their culture systems, the different technique of analysing homing ($CFSE^+$ labelled cells) and the use of $1x10^7$ nonadherent $CD34^-$ adult BM cells as accessory cells in the study by Plett *et al.,* (Plett *et al*, 2002).

Finally I did not find that culture of cells on retronectin was able to restore the homing levels of cytokine activated cells. This contrasts with an earlier observation by (Takatoku *et al*, 2001) who showed that rhesus $CD34^+$ cells transferred to culture in SCF on the CH-296 fibronectin fragment (Retronectin) after 4 days of culture in stimulatory cytokines demonstrated superior engraftment ability compared with cells cultured for 4 days in stimulatory cytokines only. My findings differ from two recent studies, one carried out by (Takatoku *et al*, 2001) who used rhesus $CD34^+$ cells and found an enhanced engraftment of cells cultured in SCF+Rn. The second study by (Glimm *et al*, 2002) used Lin^- UCB cells and used cells cultured for 96 hr in Flt3L, Steel factor, IL-3, IL-6 and G-CSF. These were then transferred to medium containing Steel factor and TPO with or without SDF-1 for 48hr, after which cells cultured in the presence of SDF-1 demonstrated enhanced ability to engraft NOD/SCID animals. These differences may relate to intrinsic differences between cord and rhesus vs adult $CD34^+$ cells and also studies by (Cashman *et al*, 2002) and (Takatoku *et al*, 2001) used engraftment in NOD/SCID mice or rhesus animals as a readout, whereas my readout was short term BM homing.

Jurkat T cells cultured in RPMI/10% FCS supplemented with FasL Plus at 5ng/ml demonstrated a significant level of apoptosis. However, pre-incubating the cells with anti human Fas mAb ZB4 of IgG_1 subclass (antagonistic) resulted in inhibition of apoptosis to levels seen with control Jurkat cells cultured in RPMI/10% FCS, demonstrating that the ZB4 mAb was functional and inhibiting apoptosis. This confirms a finding by Fadeel *et al.,* (1997), who demonstrated that the ZB4 antibody, efficiently blocked

apoptosis of Jurkat cells induced by CH-11, an agonistic Fas/CD95 mAb (Fadeel *et al*, 1997).

Freshly isolated $CD34^+$ cells and cells stimulated for 48hr with the 4 cytokine mix in the presence or absence of ZB4 generated comparable numbers of total GM-CFCs, indicating that ZB4 had no adverse effect on colony forming ability of $CD34^+$ in the methylcellulose culture. This has also been reported by Liu *et al.,* (2003) and showed that there was no difference found in the number of CFC among cells not incubated with mAb or after incubation with IgG_1 or ZB4 (Liu *et al*, 2003).

My finding in Chapter 4 and studies by Young *et al.,* (2001) and Liu *et al.,* (2003) have shown that Fas receptor expression is upregulated on both MPB and UCB $CD34^+$ cells during culture (Young *et al*, 2001;Liu *et al*, 2003). In order to determine whether increased Fas/CD95 levels caused the homing defect of cultured $CD34^+$ cells, I preincubated cytokine stimulated MPB $CD34^+$ cells with the blocking anti-Fas mAb for 30 minutes, prior to infusion into NOD/SCID mice. As control, freshly isolated $CD34^+$ cells and cells stimulated in the absence of ZB4 were also injected. I demonstrate, as in Chapter 3, that human MPB $CD34^+$ cells acquire a homing impairment in irradiated NOD/SCID mice after short term culture. In addition, I demonstrate that the homing defect was not restored by incubating cytokine stimulated MPB $CD34^+$ cells prior to transplantation with Fas/CD95 blocking mAb ZB4. My findings differ from those of Liu *et al.,* (2003) who used cord blood $CD34^+$ cells and found that the reduced homing levels of cultured CFC could be restored by incubating cells prior to transplantation with Fas/CD95 blocking mAb ZB4 (Liu *et al*, 2003). The reason for this difference is not clear but may relate to intrinsic differences between cord and adult $CD34^+$ cells and also may be related to a technical difference because $CD34^+$ cells after culture were re-purified on the Miltenyi Biotec MACS by Liu *et al.,* (Liu *et al*, 2003).

In the second approach I used sFasL to inhibit the interaction between endogenous FasL and Fas receptor, which has been suggested to have a protective role by competing against membrane-bound Fas-Ligand (Josefsen

et al, 1999). This group demonstrated that sFasL stimulation of the more mature adult human BM $CD34^+$ $CD38^+$ cells slightly increased cells death (50%) after 48hr of culture compared to cells cultured in medium alone (40%). However in striking contrast, spontaneous cell death of $CD34^+$ $CD38^-$ cells was reduced from 46% to 29% in the presence of sFasL 48hr of culture as compared to cells cultured in medium alone. This was thought to be due to the sFasL inhibiting the interaction of FasL with Fas receptor. Barcena *et al.,* (1999) showed that foetal liver $CD34^+$ $CD38^-$ cells, incubated with soluble Fas Ligand (sFasL) displayed a 2-4 fold increase in CFU and HPP-CFC formation when cultured for up to 1 week (Barcena *et al*, 1999). I demonstrate that adult MPB $CD34^+$ cells cytokine stimulated (4 days) and treated with or without sFasL generated comparable numbers of total GM-CFCs, demonstrating no adverse effect on colony formation. This difference seen by (Barcena *et al*, 1999) may be due to the longer period of culture (4 days vs 1 weeks) and also the intrinsic differences between foetal liver and adult MPB $CD34^+$ cells, as reviewed in Chapter 4.

In order to determine if blockage of Fas receptor *in vivo* with sFasL could restore homing ability of cytokine stimulated cells, I treated cytokine stimulated cells with sFasL but this did not rescue the impaired homing of cultured $CD34^+$ cells. The levels of homing of sFasL treated cells was comparable to cells cultured in the absence of sFasL, therefore both approaches I used to block Fas engagement *in vivo* were unsuccessful in reversing the homing impairment of cytokine stimulated cells, suggesting an alternative explanation for the homing defect of cytokine cultured cells.

My observations suggest that other factors apart from particular culture conditions or cell cycle status or apoptosis are likely to influence the homing and engraftment capabilities of adult HSPC. In summary, I have demonstrated in adult MPB $CD34^+$ cells that the loss of BM homing was not restored by blocking Fas ligation *in vivo*. Also altering the culture conditions for example by excluding IL-3, culturing the cells in the presence of SDF-1 and retronectin did not restore the homing defect.

Chapter 6: Effect of co-infusing non-haemopoietic stem cells and T-cells on the homing of $CD34^+$ cells in the NOD/SCID mouse model

6.1 INTRODUCTION

MSC homing and role in supporting human CD34$^+$ cell engraftment

Recent results have shown that co-transplantation of human *ex vivo* expanded MSCs together with HSCs may enhance haemopoietic recovery following a bone marrow transplantation in animal models (Nolta *et al*, 1994;Nolta *et al*, 2002;Brouard *et al*, 1998;Noort *et al*, 2002;Bensidhoum *et al*, 2004) and in humans (Koc & Lazarus, 2001;Koc *et al*, 2000;Koc *et al*, 1999). Noort *et al.,* (2002) identified a population of MSCs derived from human foetal lung, which upon co-transplantation promote engraftment of UCB CD34$^+$ cells in bone marrow, spleen, and blood of irradiated (3.5 Gy) NOD/SCID animals by mechanisms that may not require homing of MSCs to bone marrow (Noort *et al*, 2002). Angelopoulou *et al.,* (2003) showed that co-transplantation of human mesenchymal stem cells with CD34$^+$ selected MPB cells enhanced myelopoiesis and megakarypoiesis in the NOD/SCID mice (Angelopoulou *et al*, 2003). Devine *et al.,* (2001) confirmed that cultured baboon mesenchymal stem cells could home to the BM in non-human primates (Devine *et al*, 2001). Rombouts & Ploemacher, 2003 showed that primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture (Rombouts & Ploemacher, 2003). They suggest that *in vitro* propagation of BM-derived MSC dramatically decreases their homing to BM and spleen. A study by Almeida-Porada *et al.,* (2000) showed that co-transplantation of human bone marrow stromal cells increased the level of human peripheral blood donor cell expression (Almeida-Porada *et al*, 2000). These results suggest the possibility of using MSCs to promote engraftment of UCB in an attempt to accelerate the engraftment delay associated with UCB transplantation and with small grafts using adult HSC.

T cells and CD34$^+$ cell homing

Previously published reports have suggested that presence of CD8$^+$ cells led to an increase in the engraftment of CD34$^+$ cells. Martin *et al.,* (1999) showed clinical trials in humans demonstrating that the presence of CD8$^+$ cells but not CD4$^+$ cells results in an increased level of engraftment in the allogeneic transplant setting (Martin *et al*, 1999). Studies of allogeneic transplants in

mice have also shown that $CD8^+$ cells facilitate engraftment (Kaufman *et al*, 1994;Gandy *et al*, 1999;Schuchert *et al*, 2000).

A recent study showed that cord blood $CD8^+$ T cells cooperate to augment stem cell migration, homing, and engraftment (Adams *et al*, 2003). The authors demonstrate that neighbouring cells modify $CD34^+$ cell migration suggesting that combinatorial effects between cells of different types can influence cell localisation. Their results suggest that $CD8^+$ T cells contribute to stem/progenitor cell localisation through augmentation of the entry to the bone marrow microenvironment, an essential part of establishing bone marrow haemopoiesis. This model is one in which events between co-transplanted cells influence the efficiency of localisation and engraftment in haemopoietic tissues. The strategy of co-infusing $CD8^+$ T cells could be to used to enhance homing and engraftment of HSPCs. The effect seen with $CD8^+$ T cells may be similar to finding of others that have shown human bone marrow engraftment in mice can be enhanced by different accessory cell populations including irradiated mononuclear cells (Bonnet *et al*, 1999). In contrast a study by Watts *et al.,* (1995) indicated that accessory cells (lymphocytes and monocytes) reinfused with PBSC collections was not responsible for the subsequent cytokine profile or rapid haematological recovery in humans (Watts *et al*, 1995).

Chapter Aims

The aim of the present study was firstly, to isolate, expand and characterise MAPCs from postnatal BM and secondly to determine if co-transplantation of non-haemopoietic stem cells and heterologous T cells enhanced $CD34^+$ cell homing to the BM in the NOD/SCID mice. I investigated the influence of MSCs and MAPCs on short term BM homing of freshly isolated $CD34^+$ cells by co-infusion of cells at 1:1 ratio. I also determined if $CD4^+$ and $CD8^+$ cells co-infused at a 1:1 ratio enhanced the homing potential of $CD34^+$ cells to the BM in the NOD/SCID mouse model.

6.2 Special Methods

6.2.1 $CD4^+$ and $CD8^+$ cell selection

In order to obtain a homogeneous starting population of $CD4^+$ and $CD8^+$ cells, the $CD34^-$ negative fraction of a 1ml of PBSC harvest product was immunomagnetically selected using the VarioMACS LS columns (Miltenyi Biotec) in a similar manner as described for $CD34^+$ selection (Chapter 2, General Methods), except that cells were selected using anti-CD4 or anti-CD8 iron/dextran conjugated antibodies (20µl per 10^7 total cells; Miltenyi Biotec). The purity of the T cell subsets are shown in Table 6.3.

6.2.2 T cell co-culture with MPB $CD34^+$ cells

Selected $CD34^+$ cells ($2x10^6$) were placed in culture in 6 well plates at a density of $0.5x10^6$/ml in IMDM supplemented with 10% FCS and co-cultured with purified $CD4^+$ or $CD8^+$ cells at a 1:1 ratio at 37°C and 5% CO_2 for 3 hours. Cells were harvested by gentle pipetting into a 20ml universal and centrifuged at 1800 rpm for 10minutes. The cell pellet was resuspended in 400µl of HBSS, transferred to a sterile 1.5ml microfuge eppendorf tube and injected into the tail vein of sub-lethally irradiated (325 rads) NOD/SCID mice.

6.2.3 Fibronectin coated plates

Fibronectin treated plates were prepared by adding Fibronection at 100µg/ml (Sigma) to appropriate tissue culture flasks or plates for 30 minutes at room temperature.

6.2.4 MSC Isolation and characterisation

Human MSCs were obtained from the BM of healthy donors undergoing bone marrow harvests after informed consent according to the institutional guidelines under a protocol approved by the local ethics committee. Mononuclear cells were separated by centrifugation over Ficoll-Paque gradient and plated at the density of $2x10^5$ cells/cm^2 in Mesencult Culture Medium (Stem Cell Technologies). Nonadherent cells were removed after 48 hours and adherent cells cultured until they reached confluence. Cells were

trypsinised, subcultured at densities of $1x10^4/cm^2$ and immunophenotyped (see section 2.2.9) and used for experiments.

6.2.5 Isolation and culture of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells were isolated from umbilical cords by a modification of the method originally described by Jaffe *et al.,* (Jaffe *et al*, 1973). Briefly, umbilical cords were obtained within 24 hours of delivery and stored at room temperature in HBSS. The cords were checked for needle or clamp marks and any areas of damage or bruising removed. The ends of the cord were trimmed with a surgical blade and the vein of the cord at both ends were cannulated with 14 gauge intravenous cannulae and secured in place using sterile ties. The vein was flushed out using HBSS and this was continued until the cord was free from blood and the effluent ran clear. After ensuring the complete removal of the HBSS, warm collagenase (670μg/ml or 0.067% in HBSS) was inserted into the cord and the cannulae ends closed with plugs. The cord was incubated for 20 minutes at 37°C. After incubation the cord vein was flushed out with warm HBSS/1% FCS, the contents collected and centrifuged at 1000rpm for 5 minutes. The pellet was resuspend in complete culture medium (Iscove's Modified Dulbecco's Medium with 20% FCS, 50μg/ml ECGS and 20U/ml heparin). The cells were grown to confluence in $25cm^2$ Falcon tissue culture flasks pre coated with fibronectin (100μg/ml) for 30 minutes at room temperature. Once the endothelial cells had reached confluence the growth media was removed and the cells washed gently in HBSS. Warm trypsin/EDTA was added to the cells, and following incubation at 37°C for 2-5 minutes, 5-10ml of HBSS/2% FCS were added and the detached cells collected, centrifuged for 5 minutes at 1000rpm and the cell pellet resuspended in complete culture medium. The cells were then further grown to confluence in $75cm^2$ tissue culture flasks (passages 3-6) and stored in liquid nitrogen with 20% DMSO as a cryopreservant for later use.

6.2.6 Isolation, expansion and characterisation of Multipotent Adult Progenitor Cells

Human MAPCs were isolated from BM by a modification of the method originally described by Reyes *et al.,* (Reyes *et al*, 2001). BM was obtained from healthy donors at UCLH in accordance with local Ethical guidelines and processed within 4 hours of harvest collection. BMMNCs were obtained by Ficoll-Paque density gradient centrifugation. Briefly, 10ml of BM was diluted in equal volume with PBS and layered onto 20ml of Ficoll-Paque and centrifuged at 1800rpm for 30 minutes at room temperature. The mononuclear cell interface was harvested using a fine tip pasttete and the cells washed once with 10ml PBS. The cell pellet was resuspended in 1ml PBS and cell viability and counts performed. The cells were centrifuged once more at 1800rpm for 10 minutes and following aspiration of the supernatant, resuspended in anti-CD45 and anti-Gly-A iron/dextran (microbeads) conjugated antibodies (20μl per 10^7 cells) and incubated for 20 minutes at 4°C. The VarioMACs LD column, placed in its magnetic retainer, was washed once with 3ml of PBS/EDTA (CliniMACS buffer, Miltenyi Biotec) to remove residual azide. The immunolabelled cells were then washed once with 10 ml PBS w/o Ca^{++} Mg^{++} and resuspended in 1ml of PBS/EDTA and loaded onto the column. The cell suspension was allowed to run through the column and effluent collected was the depleted fraction ($CD45^-$ $Gly\text{-}A^-$ cells). The column was washed with 2 ml of PBS/EDTA and total effluent collected as depleted fraction ($CD45^-$ $Gly\text{-}A^-$ cells). Cells were then passed down a fresh column in a similar manner to maximize purity if necessary. The depleted fraction was centrifuged at 1800rpm for 15 minutes and resuspended in 200μl of MAPC expansion medium (60% low glucose DMEM, 40% MCDB-201, 1x ITS [insulin, transferrin, and selenium], 1x LA-BSA [0.5mg/ml linoleic acid, +BSA], 0.05μM dexamethasone, 1x ascorbic acid-2-phosphate [0.1mM], 2% FBS, PDGF-BB [plate-derived growth factor-BB] 10ng/ml, and EGF [epidermal growth factor] 10ng/ml) and a neat cell count performed using a haemocytometer. We plated $10x10^3$ $CD45^-$ $Gly\text{-}A^-$ cells in 200μl expansion medium into flat bottomed wells of 96 well plates pre coated with fibronectin (100μg/ml) for 30 minutes at room temperature. Cells were incubated at 37°C

and 5% CO_2 and refed every 4-6 days with MAPC expansion medium. After 7-21 days, small clusters of adherent cells developed (Figure 6.1). Once adherent cells were more than 50% confluent (usually between 2-3 weeks; clusters of about 10^3 cells, Figure 6.1), the growth medium was removed and the cells washed gently in HBSS. Warm trypsin/EDTA was added to the cells, and following incubation at 37°C for 2 minutes, DMEM low glucose/2% FBS was added and the detached cells centrifuged for 15 minutes at 1800rpm and the cell pellet resuspended in 200µl of MAPC expansion medium. A neat cell count was performed and cells replated under the same conditions at a cell density between 2-8000 cells/cm^2. Cells were further grown maintaining the cell density between 2-8000 cells/cm^2 (Table 6.1) in tissue culture treated plates (48, 24, 12 and 6 wells) and at selected intervals (passage 17, 13, 10 and 7) cells were assessed for surface phenotype and functional ability.

Table 6.1 - MAPC density maintained between 2000-8000 cells/cm^2

Plate brand	No. of wells	Growth area cm^2	Total cells per well
Nunc	96	0.33	660-2640
Costar	48	0.8	1600-6400
Costar	24	1.9	3800-15200
Costar	12	3.8	7600-30400
Costar	6	9.5	19000-76000
Nunc	T25 flask	25	50000-200000
Nunc	T80 flask	80	160000-640000

6.2.7 MAPC endothelial cell differentiation

To induce MAPC differentiation into endothelial cells, 60,000 MAPCs (passage 17, Donor 1) were replated into a fibronectin coated well (surface area =9.5 cm^2) of a Lab-Tek chamber slide (1 well permanox slide; Nalge Nunc Inc 177410) or a 6 well plate (Costar) and cultured in 2ml of MAPC basal medium (60% low glucose DMEM, 40% MCDB-201, 1x ITS [insulin, transferrin, and selenium], 1x LA-BSA [0.5mg/ml linoleic acid, +BSA], 0.05µM dexamethasone, 1x ascorbic acid-2-phosphate [0.1mM]) supplemented with VEGF at 10ng/ml. Cultures were maintained by media exchange every 4-5 days for up to 14 days at 37°C and 5% CO_2.

6.2.8 Immunocytochemistry for vWF

MAPC derived endothelial cells, HUVECs and MSCs were grown in Lab-Tek chamber slides (Nalge Nunc Inc 177410). Slide preparations were fixed in 70% methanol at –20°C for 2 minutes and washed with 2ml PBS w/o Ca^{++} Mg^{++} (PBS). Slides were blocked with 5% BSA in PBS for 2 hours at 37°C and washed three times with PBS, before overlaying with anti-vWF mAb (1:50 in PBS). After an overnight incubation in a humidified chamber at 37°C, the slides were washed again with PBS, overlayed with FITC RAM (1:200 in PBS), and incubated for a further 2 hours at room temperature. After a final 3 washes slides were mounted in Vectashield (Vector Laboratories) and viewed under a fluorescent microscope.

6.2.9 In vitro vascular tube formation

A 10ml vial of Matrigel basement membrane matrix (BD) was thawed on ice and using a cooled 1ml glass pippette (Volac) mixed to homogeneity. A 24 well tissue culture plate (Costar) was placed on ice for 5 minutes and then 500µl of Matrigel was added to each well. The plate was then incubated at 37°C for 3 hours. After 3 hours each well was washed once with 0.5ml HBSS and 60,000 cells (MAPC-derived endothelial cells, HUVECs, and Mesenchymal Stem Cells) seeded into each well in a total volume of 0.5ml medium (MAPC-derived endothelial cells cultured in basal medium plus VEGF 10ng/ml; HUVECs cultured in HUVEC culture medium; MSCs cultured in MSC growth medium). The plate was incubated overnight at 37°C and 5% CO_2 and vascular tube formation examined the following morning on an inverted microscope.

6.2.10 Ac-LDL uptake

Cells were grown in a 6 well tissue culture plate (as detailed above) and incubated with Dil-Ac-LDL at 10µg/ml (Human low density lipoprotein acetylated and labelled with the fluorescent probe, 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (Dil) Tebu-Bio) in the cell growth medium overnight at 37°C and 5% CO_2. The following morning the Dil-Ac-LDL medium was removed and cells washed once with 2ml of HBSS. Cells

were visualised with an Olympus IX70 inverted fluorescence microscope (Olympus) using a standard rhodamine excitation/emission filter. The fluorescent label, DiI is internalised and the cell remains viable, therefore labelled cells can be trypsinised and be reseeded.

6.2.11 MSC and MAPC co-injection with MPB $CD34^+$ cells

Selected $CD34^+$ cells ($2x10^6$) were transplanted in the presence or absence of MSCs (1 million) and MAPCs (1, 0.5 and 0.2 million). Cells were resuspended in 400µl of HBSS, transferred to a sterile 1.5ml microfuge eppendorf tube and injected into the tail vein of sub-lethally irradiated (325 rads) NOD/SCID mice.

6.3 RESULTS

6.3.1 Isolation, expansion and characterisation of MAPCs from postnatal BM

6.3.1.1 Culture of undifferentiated MAPCs and morphology

Normal BMMNCs were depleted of $CD45^+$ and $GlyA^+$ cells using the MACs immunomagnetic beads. $CD45^-$ $GlyA^-$ cells were plated on fibronectin coated 96 well plate in expansion medium with 2% FBS, 10ng/ml EGF, and 10ng/ml PDGF-BB. As shown in Figure 6.1, panel 1-3, small clusters of adherent cells developed between 7-21 days after culture. When clusters appeared of approximately 1000 cells, cells were passaged and replated under the same culture conditions. Cell densities were maintained between 2-8000 cells/cm^2 (Figure 6.1, panel 5). MAPCs cultured at a low density and with 2% FBS were small and have scant cytoplasm with few vacuoles and granules. The cell growth rate was monitored for 4 BM donors and the cell doubling time varied from donor to donor and was between 40-60 hr (Figure 6.2). The population doublings was 5, 3, 6 and 7 for donors 1, 2, 3 and 4, respectively.

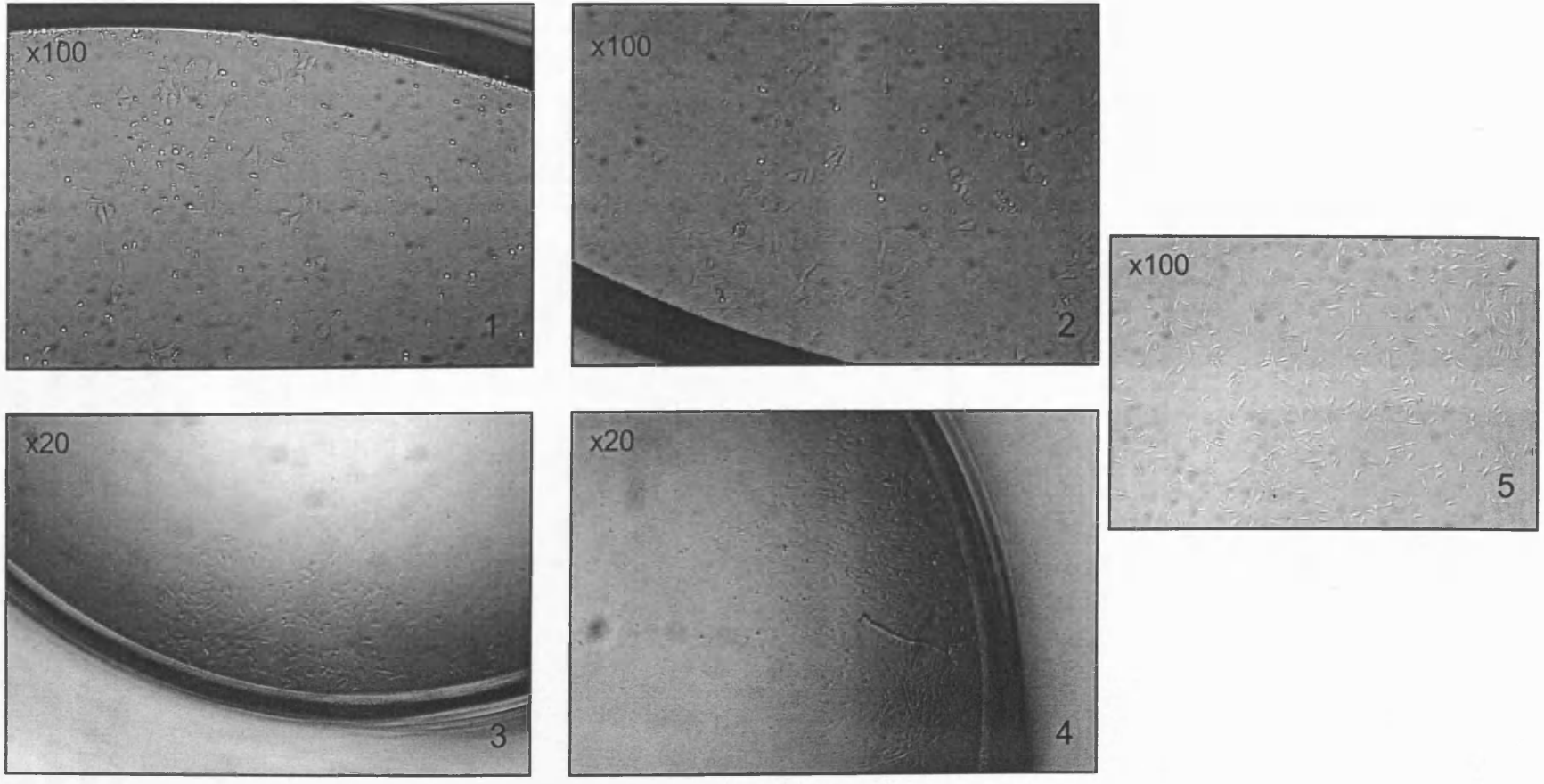


Figure 6.1 - Morphology of undifferentiated MAPCs. $CD45^-$ $GlyA^-$ cells were plated in 96 well plates (1-4) on Fn in expansion medium with 2% FBS (Hyclone ES screened), 10ng/ml EGF, and 10ng/ml PDGF-BB and passaged as detailed in Chapter 6 Special Methods. After 7-21 days, small clusters of adherent cells developed (panel 1-3) and when clusters appeared of approximately 1000 cells, cells were detached and replated (panel 5) into a 48 well plate and maintained at a cell density of $2\text{-}8x10^3/cm^2$. A representative example is shown (Donor 1). Cells were analysed by bright-field microscopy. Shown are bright-field microscopy pictures at magnification as indicated in each plate.

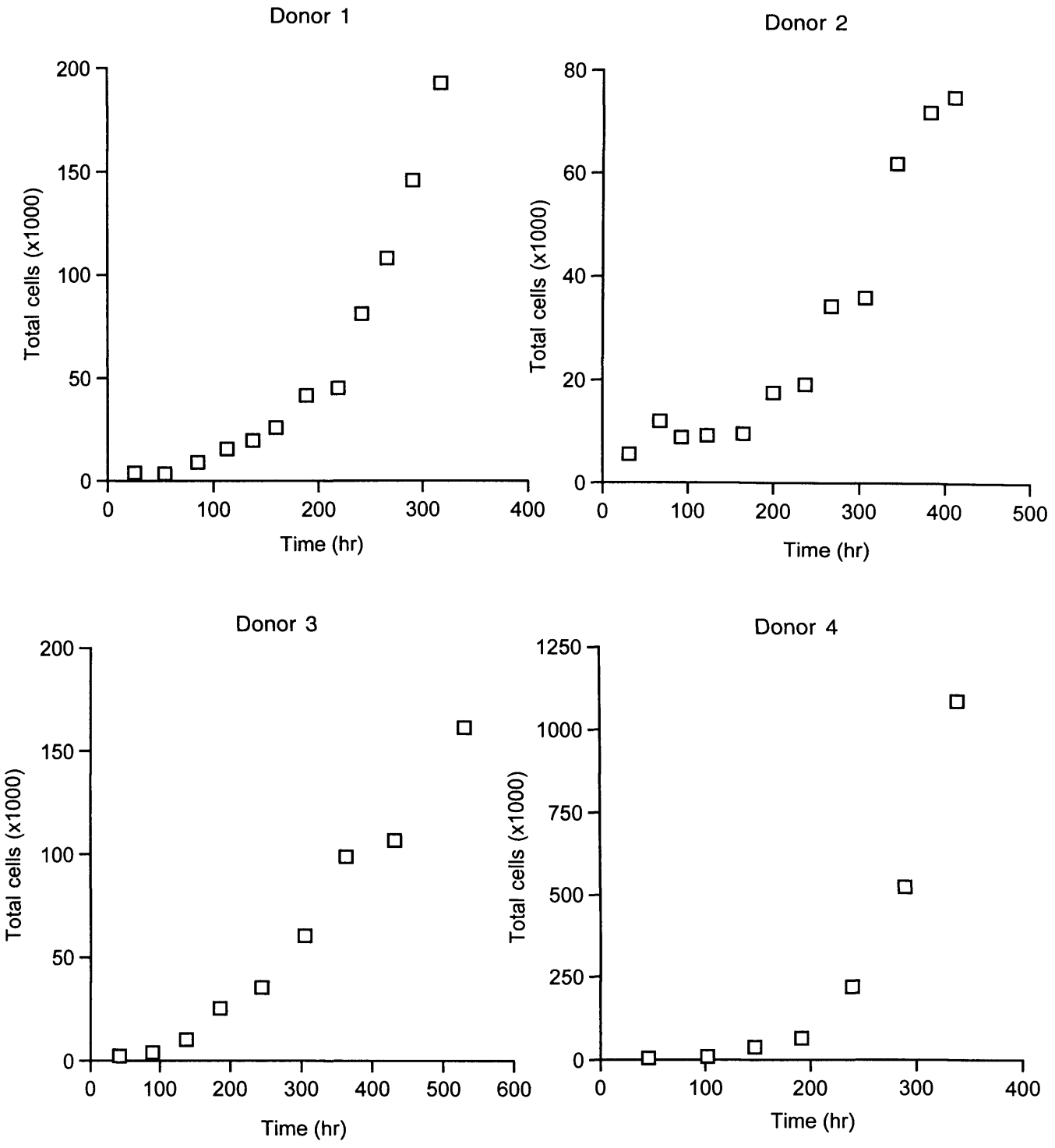


Figure 6.2 - MAPC cell growth curve. CD45$^-$ Gly A$^-$ cells were plated on Fn in expansion medium with 2% FBS, 10ng/ml EGF, and 10ng/ml PDGF-BB as detailed in Chapter 6 Special Methods and passaged at time points indicated. Cells were maintained between 2-8000 cells/cm^2. Cells were counted at each passage with a haemocytometer. The data is from a single normal BM donor.

6.3.1.2 Immunophenotypic analysis of undifferentiated MAPCs

Undifferentiated MAPCs were generated as outlined above and immunophenotyped by flow cytometry. Cell phenotype was performed on 4 normal BM donors at passages 17, 13, 10 and 7. Undifferentiated MAPCs did not express CD34, CD45 and HLA-DR (Table 6.2). Cells expressed high levels of CD13, SH2, SH4, HLA-ABC, CD44 and intermediate levels of CD49b (Table 6.2).

Table 6.2 – MAPC cell surface antigen profile

	% positive cells			
Antigen	Donor 1	Donor 2	Donor 3	Donor 4
CD45	0	4	0	6
CD34	0	0	0	0
CD13	98	94	82	99
SH2	99	-	-	69
SH4	97	-	-	96
HLA-DR	0	-	-	-
HLA-ABC	99	-	-	-
CD49b	-	39	-	37
CD49d	-	-	-	18
CD44	-	82	-	79

BM MNC $CD45^-$ $GlyA^-$ cells were plated on Fibronectin coated plates in expansion medium with 2% FBS, 10ng/ml EGF, and 10ng/ml PDGF-BB as described in Chapter 6 Special Methods. Cells were harvested and labelled with antibodies directly conjugated to the flurochromes FITC or PE and analysed by flow cytometry. Cell phenotype was performed at passages 17, 13, 10 and 7 for Donors 1, 2, 3 and 4 respectively. Data are given for 4 normal BM donors and isotype control staining has been subtracted. (- not determined)

6.3.2 Functional characterisation of MAPC derived endothelial cells

6.3.2.1 Human MAPCs differentiate into cells with phenotypic characteristics of endothelium

Undifferentiated MAPCs generated as described above from normal BM (Donor 1), were induced to differentiate into endothelial cells. MAPC derived endothelial cells were plated at a high confluence (60000 cells/well) in 6 well plate Fibronectin coated and grown in expansion medium without serum and EGF, PDGF-BB, but with VEGF at 10ng/ml for 14 days. MAPC-derived

endothelial cells were replated on Fibronectin coated chamber slides. Cells were then stained with antibodies against vWF (FITC) by immunocytochemistry. vWF staining is found throughout the cytoplasm of MAPC-derived endothelial cells (Figure 6.3, panel a). Primary human endothelial cells (HUVECs) served as a positive control and stained strongly for vWF throughout the cytoplasm (Figure 6.3, panel b). In contrast MSCs were negative for vWF staining (Figure 6.3, panel c).

6.3.2.2 Functional characteristics of MAPC derived endothelial cells

A functional characteristic of endothelial cells is that they take up LDL (Steinberg *et al*, 1985). This was tested by incubating MAPCs induced to differentiate with VEGF for 14 days with Dil-Ac-LDL overnight. Dil uptake was very strong in primary human endothelial cells and staining was seen throughout the cytoplasm in majority of the cells (Figure 6.4, panel b). The uptake of Dil was not as strong in MAPC derived endothelial cells but cells could be seen with Dil staining throughout the cytoplasm (Figure 6.4, panel a). In contrast MSCs did not take up Dil and were negative for Dil staining (Figure 6.4, panel c).

Another characteristic of endothelial cells is the ability to form vascular tubes when plated on Matrigel. As shown in Figure 6.5, culture of primary human endothelial cells (HUVECs) on matrigel resulted in vascular tube formation with a complex lattice pattern (Figure 6.5, panel b). MAPC-derived endothelial cells formed vascular tubes but not the complex lattice pattern seen with primary endothelial cells (Figure 6.5, panel a). In contrast MSCs did not form any vascular tubes (Figure 6.5, panel c).

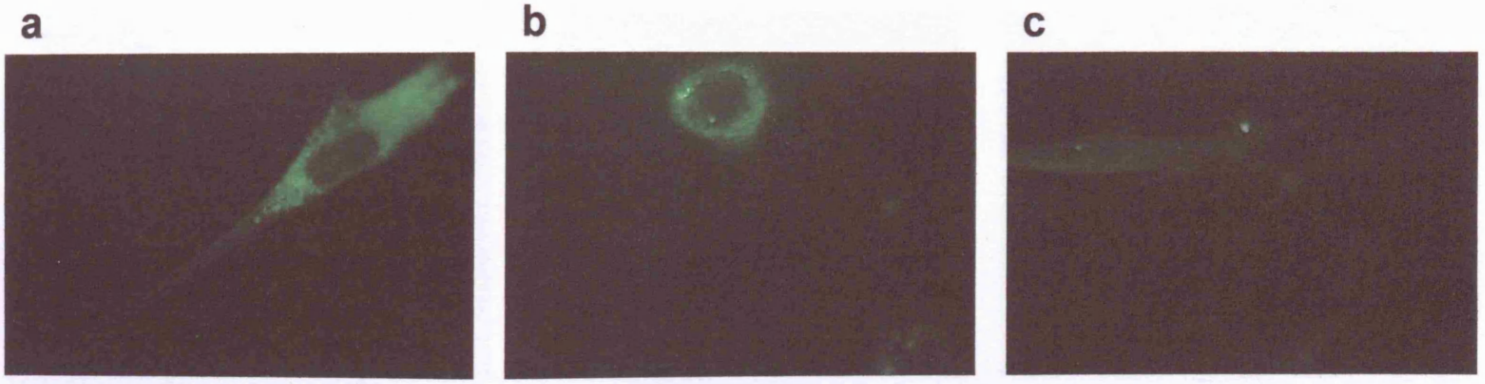


Figure 6.3 - von Willebrand factor expression by MAPC-derived endothelial cells. Undifferentiated MAPCs were plated on Fibronectin coated 6 well plate (60000 cells/well) in expansion medium without serum, EGF or PDGF-BB, but with VEGF 10ng/ml for 14 days. MAPC-derived endothelial cells were replated on fibronectin coated chamber slides. Cells were then stained with antibodies against vWF (FITC) and analysed by confocal microscopy. vWF staining is found throughout the cytoplasm of MAPC-derived endothelial cells (a) and HUVECs (b), but absent in MSCs (c). Representative example is shown (Donor 1). HUVECs served as a positive control.

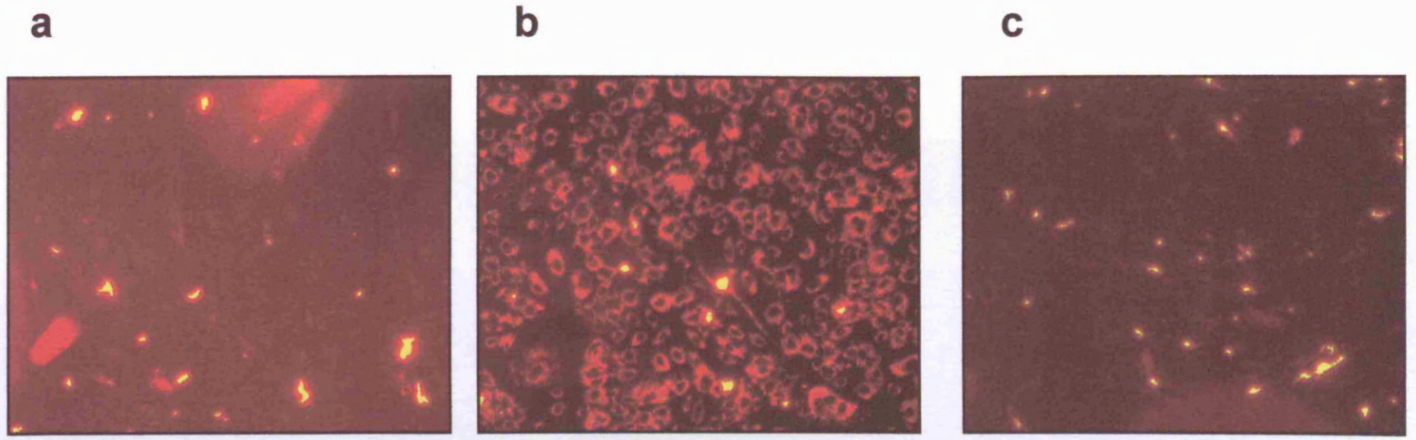


Figure 6.4 - Ac-LDL uptake by MAPC-derived endothelial cells. Undifferentiated MAPCs were plated on Fibronectin coated 6 well plate (60000 cells/well) in expansion medium without serum, EGF or PDGF-BB, but with VEGF 10ng/ml for 14 days. Cells were incubated overnight with Dil-Ac-LDL and analysed by confocal microscopy. Dil uptake was found in both MAPC-derived endothelial cells (a) and HUVECs (b), but absent in MSCs (c). Representative example is shown (Donor 1). HUVECs served as a positive control.

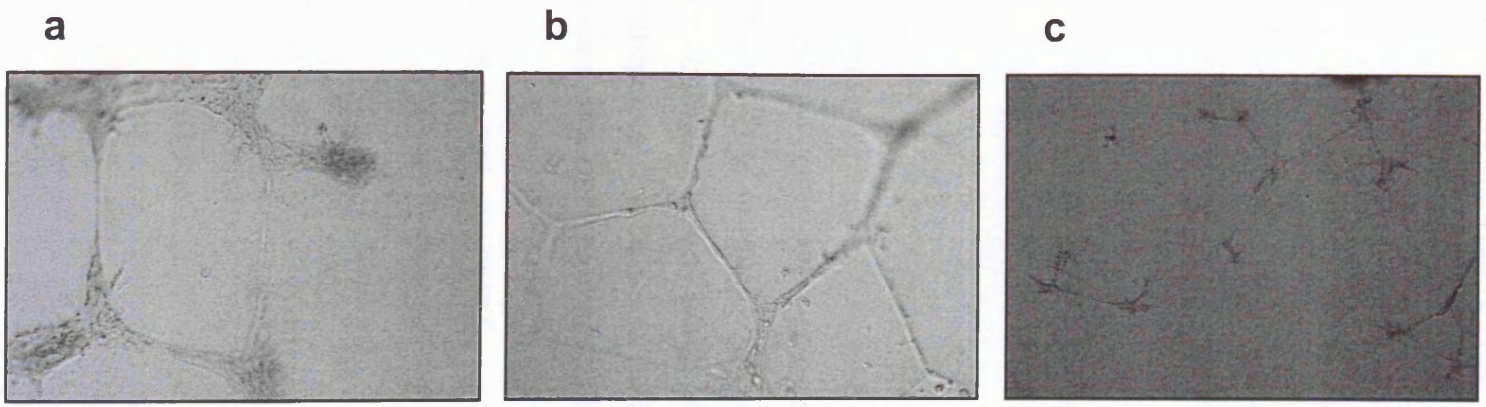


Figure 6.5 - Vascular tube formation by MAPC-derived endothelial cells. Undifferentiated MAPCs were plated on Fibronectin coated 6 well plate (60000 cells/well) in expansion medium without serum, EGF or PDGF-BB, but with VEGF 10ng/ml for 14 days. Cells were then replated in Matrigel with VEGF 10ng/ml. After overnight incubation at 37°C, typical vascular tubes could be seen in both MAPC-derived endothelial cells (a) and HUVECs (b), but absent in MSCs (c). Representative example is shown (Donor 1). HUVECs served as a positive control. Cord formation was analysed on a confocal microscope

6.3.2.3 Immunophenotypic analysis of MSCs

Bone marrow stromal cells were isolated from normal BM donors and expanded in specially designed Mesencult culture medium for 2 days prior to removal of nonadherent cells. Initially a heterogeneous adherent cell layer including fibroblast-like cells were observed which changed after few passages to a uniform spindle shaped population. These cells were immunophenotyped by flow cytometry. MSCs did not express the haemopoietic markers CD34 (2.9%) and CD45 (2.5%) but expressed high levels (>99%) of SH2, SH4 and CD13.

6.3.3 Influence of MSCs and MAPCs on short term BM homing of freshly isolated $CD34^+$ cells

6.3.3.1 Effect of MSC and MAPCs on CFC numbers

In order to exclude possible roles of MSCs and MAPCs on colony formation, fresh $CD34^+$ cells were cultured in the presence or absence of MSCs and MAPCs. The number of assayable progenitors was determined by methylcellulose colony assay. As shown in Table 6.3, $CD34^+$ cells cultured in the presence of MSCs and MAPCs generated slightly higher numbers of total colonies with comparison to $CD34^+$ cells alone.

Table 6.3 - Effect of MSCs and MAPCs on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells			
	Donor 1	Donor 2	Donor 3	Donor 4
$CD34^+$	102	25	48	63
$CD34^+$/MSC	160	69	70	72
$CD34^+$/MAPC	137	15	N/A	68

Freshly isolated $CD34^+$ cells in the presence or absence of MSCs and MAPCs was assessed in methylcellulose culture for colony forming cells. Data are expressed per 1000 cells plated and given for 4 normal BM donors.

6.3.3.2 Effect of MSC or MAPCs co-infused with CD34$^+$ cells on short term BM homing

In order to assess the influence of MSCs and MAPCs on short term BM homing, MSCs and MAPCs were co-infused with CD34$^+$ cells and CFC homing to the BM quantified. The BM of animals transplanted with CD34$^+$ cells in the presence or absence of MSCs and MAPCs was recovered at 24hr post-infusion and single cell suspensions plated into semisolid culture. As shown in Figure 6.6, CFC from CD34$^+$/MSC transplanted animals showed a small increase (median 0.84, range 0.5-1.63%) in BM homing compared with control animals transplanted with CD34$^+$ cells alone (median 0.56, range 0.38-1.21%) however, this was not statistically significant (P value 0.2416, Mann-Whitney U test). The homing levels of CFC to the BM of CD34$^+$/MAPC transplanted mice (median 0.22, range 0.16-1.2%) was comparable to control mice infused with CD34$^+$ cells only.

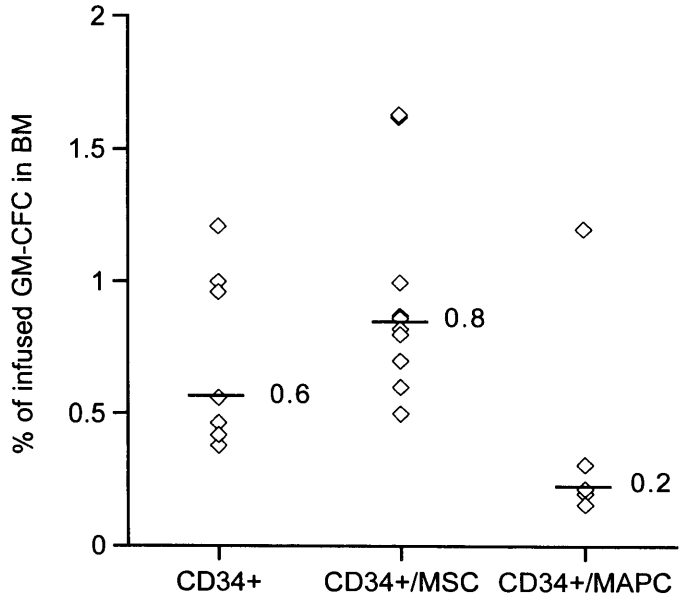


Figure 6.6 - Effect of MSCs and MAPCs on homing of MPB CD34$^+$ cells in the NOD/SCID mice. Freshly isolated MPB CD34$^+$ cells (2 million) were transplanted alone or in the presence of MSCs (1 million) and MAPCs (1, 0.5 and 0.2 million). Short term BM homing was assayed 24hr post transplantation. The data are from 4 independent experiments, each data point represents 1 animal and numbers and bars indicate the medians.

6.3.4 Role of T cell subsets

6.3.4.1 Isolation of $CD4^+$ and $CD8^+$ cells

The CD34-negative cell fraction was selected for $CD4^+$ and $CD8^+$ cells using the immunomagnetic VarioMACS system as described in Chapter 6 Special Methods. I analysed by flow cytometry the purity of the isolated cells by staining the cells with a fluorochrome conjugated anti- human CD4 and CD8. As shown in Table 6.4, the purity of $CD4^+$ cells and $CD8^+$ cells was 88 ± 6% (n=3) and 96 ± 2% (n=4), respectively. Both cell fractions were strongly positive for CD3 (>90%), as expected.

Table 6.4 - Purity of $CD4^+$ and $CD8^+$ cells from adult mobilised peripheral blood (MPB). $CD4^+$ and $CD8^+$ cells were positively selected from $CD34^-$ cell fraction using the immunomagnetic VarioMACS system as described in Chapter 6 Special Methods. Purified cell fractions were stained with RD1 and FITC-conjugated anti-human CD4 and CD8 respectively, and analysed by flow cytometry. Data are presented of % positive cells for 4 independent experiments.

	Purity (%)	
Experiment No.	$CD4^+$ cells	$CD8^+$ cells
1	83	95
2	95	99
3	N/D	95
4	85	97
Mean ± SD	88±6	96±2

6.3.4.2 Effect of $CD4^+$ and $CD8^+$ cells on CFC numbers

In order to determine the effects of $CD4^+$ and $CD8^+$ cells on colony forming activity. I assessed the functional activity of freshly isolated haemopoietic progenitors cultured for 3hr in IMDM/10% FCS alone or with purified $CD4^+$ and $CD8^+$ cells at 37°C and 5% CO_2 in the methylcellulose culture. As shown in Table 6.5, $CD34^+$ cells cultured in the presence of $CD4^+$ and $CD8^+$ cells generated comparable numbers of total colonies compared with control $CD34^+$ cells alone, n=3.

Table 6.5 - Effect of T cell subsets on colony forming cells (CFCs)

	No. of GM-CFC per 1000 cells		
	Expt 1	Expt 2	Expt 3
Fresh	50	98	102
$CD34^+/CD4^+$	52	88	98
$CD34^+/CD8^+$	58	119	80

Freshly isolated $CD34^+$ cells were cultured in IMDM/10% FCS alone (fresh) or with purified $CD4^+$ and $CD8^+$ cells at a 1:1 ratio at 37°C and 5% CO_2 for 3 hr. Colony forming activity of cells was assessed in methylcellulose culture. Data are expressed per 1000 cells plated and given for 3 independent experiments.

6.3.4.3 Effect of $CD4^+$ and $CD8^+$ cells on in vivo homing of $CD34^+$ cells to the BM

Freshly isolated haemopoietic progenitors and purified $CD4^+$ and $CD8^+$ cells were co-cultured for 3 hr in Iscoves/10% FCS and were infused by the tail vein injection into NOD/SCID mice. Mononuclear cells recovered from murine BM 24 hr post transplantation were plated into semisolid culture for enumeration of human CFC, as described in Chapter 2 General Methods. Aliquots of pre infusion $CD34^+$ cells were set up in parallel methylcellulose culture and used as reference in order to quantify homing efficiency of CFC. As shown in Figure 6.7, $CD34^+$ cells co-infused with $CD4^+$ cells resulted in similar levels of CFC homing to the BM compared with control $CD34^+$ cells alone (GM-CFC mean of 0.98, range 0.75-1.19% and 1.17, range 0.62-1.57% for $CD34^+$ only and $CD34^+$ co-infused with $CD4^+$ cells, respectively), n=3. Co-infusion of $CD8^+$ cells slightly enhanced the homing to the BM (mean 1.3, range 0.74-1.59%), but this was not statistically significant (P value 0.1229, Mann-Whitney U Test). To determine if this modest enhancement of $CD34^+$ cells homing was distinct to the BM environment, homing to the spleen was evaluated (Table 6.6). Consistent with that seen in the marrow, very similar levels of $CD34^+$ cell homing was noted when $CD8^+$ cells or $CD4^+$ cells were co-infused (Table 6.6), n=3. As shown in Table 6.6, in experiment 3, fresh $CD34^+$ transplanted mice formed 42 and 38 GM-CFC colonies per 10^6 murine spleen cells, whereas $CD4^+$ and $CD8^+$ admixed transplanted mice formed 44, 40 and 41, 36 GM-CFC colonies per 10^6

murine spleen cells, respectively. Saline control and irradiation control mice grew no colonies.

Thus, no consistent effect of co-infusing MSCs, MAPCs or $CD8^+$ T cells on short term BM homing of freshly isolated $CD34^+$ cells was observed in the NOD/SCID mouse model.

Table 6.6 - Homing of MPB $CD34^+$ cells cultured with T cell subsets to the spleen.

	No. of GM-CFC per $1x10^6$ cells		
	Expt 1	Expt 2	Expt 3
Fresh	82, 14	122, 140	42, 38
$CD34^+/CD4^+$	42, 4	156, 225, 58	44, 40
$CD34^+/CD8^+$	56, 71	112, 71, 58	41, 36
Saline	N/D	0	N/D
Irradiation ctl	0	N/D	N/D

Freshly isolated $CD34^+$ cells were cultured in IMDM/10% FCS alone (fresh) or with purified $CD4^+$ and $CD8^+$ cells at a 1:1 ratio at 37°C and 5% CO_2 for 3 hr. Homing of cells to the spleen at 24hr post transplantation is shown. Each data point represents a animal which were sub-lethally irradiated and infused with $2x10^6$ cells of each population by tail vein injection. Data are presented per million spleen cells and from three independent experiments.

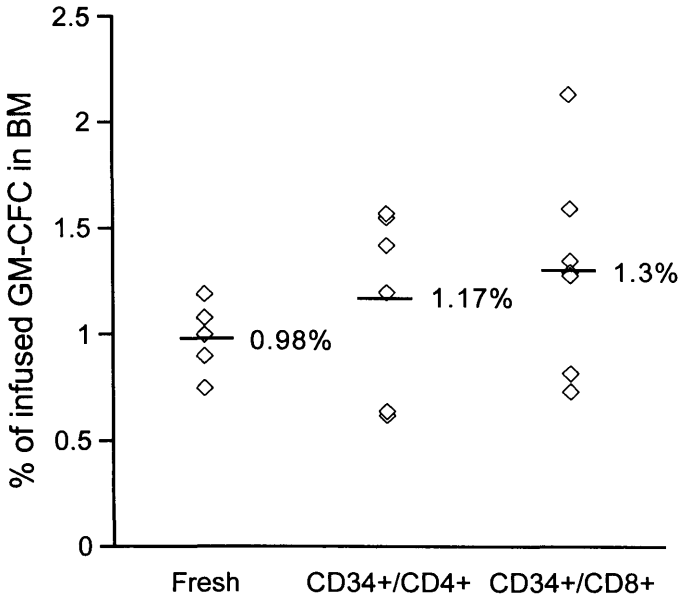


Figure 6.7 - Effect of T cell sub-population on homing of CD34$^+$ cells in the NOD/SCID mice. Freshly isolated MPB CD34$^+$ cells were cultured in IMDM/10% FCS alone and with purified CD4$^+$ (purity >83%) or CD8$^+$ (purity 95%) cells at a 1:1 ratio at 37°C for 3hr, before transplantation into NOD/SCID animals (2 million cells of each population per animal). Short term BM homing was assayed 24hr post transplantation. The data are from 3 independent experiments, each data point represents 1 animal and numbers and bars indicate the means.

6.4 DISCUSSION

MSC characterisation

MSCs can be readily obtained from adult BM samples and rapidly expanded *ex vivo*. MSCs did not express the haemopoietic markers CD34 and CD45 but expressed high levels of SH2 (CD105) and SH4 (CD73) and this concurs with surface antigenic properties of MSC described by others (Deans & Moseley, 2000).

MAPC characterisation

MAPCs isolated and expanded from normal adult BM donors in 2% FBS were small cells with scant cytoplasm with few vacuoles and granules. This morphology remained unchanged as long as cells were maintained at cell density of 2-8000 cells/cm^2. Similar morphology of MAPCs has been documented by (Reyes *et al*, 2001). The cell doubling time was ranged from 40-60 hr, similar to reports by Reyes *et al.,* (Reyes *et al*, 2001).

Immunophenotypic analysis by flow cytometry of undifferentiated MAPCs showed that cells did not express CD34, CD45 and HLA-DR. Cells expressed high levels of CD13, SH2, SH4, HLA-ABC, CD44 and intermediate levels of CD49b. My findings differ from those of Reyes *et al.,* (2001) who immunophenotyped cells obtained after 10-15 cell doublings and showed that the cells did not express CD10, CD31, CD34, CD36, CD38, CD50, CD62E, CD106, CD117, H1P12, fibroblast surface antigen (1B10), HLA-DR, HLA-ABC, CD45, Tie, Tek. Cells expressed low levels of β2microglobulin, CD44 (Hyaluronic acid receptor), CDw90, KDR, Flt1 and high levels of CD13 and CD49b (Reyes *et al*, 2001). The reason for these differences in HLA-ABC, CD44 and CD49b (VLA-2) expression are not clear but may relate to unintentional differences in culture conditions. Reyes *et al.,* (2001) showed that MPCs cultured at low density and with 2% or lower FCS are class I-HLA$^-$ and HLA-DR$^-$ and express only low levels of CD44. Culture with greater than 2% FCS or culture at high density (greater than 8000 cells/cm^2) yielded cells that were significantly larger and more vacuolated, and caused acquisition of high levels of of CD44, HLA-DR and Class I HLA

and decreased levels of CD13 and CD49b (Reyes *et al*, 2001). The phenotype I report here for MAPCs, was similar to the phenotype seen when cells were cultured with greater than 2% FCS or culture at high density (greater than 8000 cells/cm^2), although I expanded the cells with 2% FCS and maintained the cells between 2-8000 cells/cm^2. The phenotype of undifferentiated MAPCs I generated, however, were similar to the phenotype reported for MSCs (Colter *et al*, 2000;Gronthos *et al*, 1994;Pittenger *et al*, 1999). MSCs have only been shown to be capable of making a handful of tissues such as bone, cartilage, fat and muscle (Pittenger *et al*, 1999;Mackay *et al*, 1998). Recently, (Lodie *et al*, 2002) have shown that a systematic analysis of reportedly distinct populations of multipotent bone marrow derived stem cells (MAPCs vs MSCs) reveals a lack of distinction between them. Each cell, for example, expressed the same or similar cell surface markers, or antigens. They also showed common ability to undergo differentiation into nerve, cartilage, muscle, and endothelial cells based on culture conditions. There is also debate over a seemingly small, but potentially important change in the method that (Lodie *et al*, 2002) used to obtain MAPCs. A key step in obtaining MAPCs, according to (Reyes *et al*, 2001) is to grow bone marrow cells at a very low density. Yet (Lodie *et al*, 2002) obtained nothing this way and instead grew cells at a high concentration.

In order to investigate this further, the undifferentiated MAPCs generated were tested for their ability to differentiate into cells of viceral mesodermal origin, such as endothelial cells. When this was tested, there was no evidence that MSCs cultured under conditions initially developed by (Pittenger *et al*, 1999) differentiate into cells with endothelial characteristics (Reyes *et al*, 2001). However, very recently Oswald *et al.,* (2004) have shown that by culturing confluent MSCs in low-serum (2% FCS) supplemented with VEGF (50ng/ml), the MSCs acquire several features of mature endothelium, including the expression of VEGF receptors (KDR and FLT1), VE-cadherin, VCAM-1, and vWF. They showed also an enhanced ability to form capillary-like structures in semi-solid medium (Oswald *et al*, 2004).

Reyes *et al.,* (2002) demonstrated that MPCs from BM have the ability to differentiate into endothelial cells that have *in vitro* functional characteristics that are indistinguishable from those of mature endothelial cells (Reyes *et al*, 2002). I demonstrated not only that MAPCs differentiate into cells that express markers of endothelial cells, such as vWF, but show that VEGF induced MAPCs to function like endothelial cells. MAPC derived endothelial cells, like primary endothelial cells such as HUVECs, have the ability to takeup LDL and form vascular tubes in an *in vitro* matrigel assay, although there were subtle differences between MAPC derived endothelial cells and primary endothelial cells (HUVECs). In comparison MSCs do not express vWF and do not takeup LDL and form cords on an extracellular matrix. The above data demonstrate that the undifferentiated MAPCs I generated are different to MSCs, in that undifferentiated MAPCs could be induced to differentiate into endothelial cells. However, very recently it has been shown that MSCs can be differentiated into endothelial cells *in vitro* (Oswald *et al*, 2004).

Effect of co-transplantation of MSCs and MAPCs on CD34$^+$ cell homing

Recent results have shown that co-transplantation of human *ex vivo* expanded MSCs together with HSCs enhances haemopoietic recovery following a bone marrow transplantation in animal models (Nolta *et al*, 1994;Nolta *et al*, 2002;Brouard *et al*, 1998;Noort *et al*, 2002;Bensidhoum *et al*, 2004) and in humans (Koc & Lazarus, 2001;Koc *et al*, 2000;Koc *et al*, 1999). Noort *et al.,* (2002) identified a population of MSCs derived from human foetal lung, which upon co-transplantation promote engraftment of UCB CD34$^+$ cells in bone marrow, spleen, and blood however, these MSC do not appear to home to bone marrow (Noort *et al*, 2002). Angelopoulou *et al.,* (2003) showed that co-transplantation of human mesenchymal stem cells with CD34 selected MPB cells enhances myelopoiesis and megakarypoiesis in the NOD/SCID mice (Angelopoulou *et al*, 2003). These results suggest the possibility of using MSCs to promote engraftment of UCB cells in an attempt to accelerate the engraftment delay associated with UCB transplantation.

Because co-transplantation of marrow derived stromal cells has been shown to enhance engraftment of human UCB HSCs, we hypothesised this may be mediated at the level of initial homing of $CD34^+$ cells to the BM of NOD/SCID mice. We tested this hypothesis by transplanting MPB $CD34^+$ selected cells into sublethally irradiated NOD/SCID mice with or without culture expanded MSCs or MAPCs and evaluated the homing ability to the BM by enumeration of human CFCs.

$CD34^+$ cells cultured in the presence of MSCs or MAPCs generated slightly higher numbers of total colonies with comparison to $CD34^+$ cells alone. Therefore the presence of MSCs or MAPCs in the CFC assay had no significant effect on $CD34^+$ cell colony forming activity. I demonstrate that $CD34^+$ cells co-transplanted with MSCs in the NOD/SCID mice showed a small increase in BM homing compared with control animals transplanted with $CD34^+$ cells alone, however, this was not statistically significant (P value 0.1229, Mann-Whitney U test). In contrast the homing levels of CFC to the BM of $CD34^+$/MAPC transplanted mice was comparable to control mice infused with $CD34^+$ cells only. A similar finding was reported by Noort *et al.,* (2002) who examined the possibility that homing of UCB $CD34^+$ cells was affected by co-transplantation of MSC (Noort *et al*, 2002). These authors determined the percentage of $CD45^+$ cells in the BM, PB, lungs, spleen, and liver by flow cytometry 24hr after transplantation of $CD34^+$ cells (0.5-1 million) in the presence or absence of MSCs (5 million). They found no difference in the percentage of the injected UCB $CD34^+$ cells in the BM, PB, lungs, liver and spleen of mice transplanted in the presence or absence of MSCs (Noort *et al*, 2002). They concluded that homing of $CD34^+$ cells seems not to be affected by co-transplantation of MSC (Noort *et al*, 2002).

My finding reported here, together with previous reports that co-transplantation of human *ex vivo* expanded MSCs hastens haemopoietic recovery following a bone marrow transplantation in animal models (Nolta *et al*, 1994;Nolta *et al*, 2002;Brouard *et al*, 1998;Noort *et al*, 2002;Bensidhoum *et al*, 2004) and in humans (Koc & Lazarus, 2001;Koc *et al*, 2000;Koc *et al*, 1999). Suggests that the engraftment benefit seen with co-transplanted

MSCs may be independent of an effect on $CD34^+$ cell homing to the BM. Noort *et al.,* (2002) concluded that upon co-transplantation, MSC, but not irradiated $CD34^-$ or B cells, promote engraftment of UCB $CD34^+$ cells in bone marrow, spleen, and blood by a mechanism that may not require homing of MSC to the BM (Noort *et al*, 2002). The ability of MAPCs to enhance homing has not been studied to date, but I demonstrate that these cells do not significantly increase the homing ability of adult MPB $CD34^+$ cells in the NOD/SCID mice model.

The CD34 negative cell fraction positively selected for $CD4^+$ and $CD8^+$ cells yielded a purity of 88% and 96%, respectively. This level of purity is comparable to the report by (Adams *et al*, 2003). $CD34^+$ cells cultured in the presence of $CD4^+$ and $CD8^+$ cells generated comparable numbers of total colonies compared with control $CD34^+$ cells alone. Therefore the presence of $CD4^+$ or $CD8^+$ cells does not adversely affect CFC formation.

Adams *et al.,* (2003) have reported that UCB $CD34^+$ cells admixed with $CD4^+$ cells consistently engrafted less well than those admixed with $CD8^+$ cells (Adams *et al*, 2003). $CD34^+$ cells admixed with $CD8^+$ cells engrafted with a mean 10 fold increase over control $CD34^+$ cells. This enhanced engraftment correlated with the finding that $CD8^+$ cells significantly enhanced the homing to the BM of CFDA-SE labelled $CD34^+$ cells. I was unable to demonstrate that either $CD4^+$ or $CD8^+$ cells co-infused with adult MPB $CD34^+$ cells enhanced the homing to the BM. $CD34^+$ cells admixed with $CD8^+$ cells showed slightly higher homing to the BM compared with $CD34^+$ cells injected alone or $CD34^+$ cells admixed with $CD4^+$ cells, this did not reach statistical significance (P value 0.1229, Mann-Whitney U test). My findings differ from those of Adams *et al.,* (2003) who used UCB $CD34^+$ cells labelled with the CFDA-SE dye and found that $CD8^+$ cells enhanced homing to the BM (Adams *et al*, 2003). The reasons for this difference are not clear but may relate to intrinsic differences between cord and adult $CD34^+$ cells (UCB vs MPB) and also differences in the assay used to study homing. Adams *et al.,* (2003) studied homing to the BM using direct examination of fluorescently labelled (CFDA-SE) cells *in vivo* (Adams *et al*, 2003). In contrast I used the

CFC assay to determine homing to the BM. It is also now well established that CB lymphocytes are more naïve than adult lymphocytes. The evidence for this, comes from the observation that the majority of CB lymphocytes express CD45RA, whereas the majority of adult lymphocytes are CD45RO (Bradstock *et al*, 1993;Keever, 1993;Bofill *et al*, 1994). Adult and CB cells also exhibit differences in cytokine production and in general it has been noted that the frequency of T cells with the ability to produce TNFα, IFNγ, IL-2, IL-4 or IL-10 is reduced in CB compared to adult blood (reviewed in (Cohen *et al*, 1999). Thus, CB $CD8^+$ cells may function differently in the homing assay when compared with adult $CD8^+$ T cells. Another difference is the strains of mice used, I used the NOD/SCID strain while Adams and co-workers used the more sensitive and recently developed β2m NOD/SCID strain. The β2m knockout NOD/SCID mice in addition to the B and T cell defect and defects in the complement pathway and macrophage function in NOD/SCID mice, also lack natural killer (NK) cells and thus facilitate multilineage differentiation of 10-fold fewer human CB cells than required to achieve comparable levels of engraftment in NOD/SCID hosts (Kollet *et al*, 2000). To determine if this enhancement of $CD34^+$ cell localisation was distinct for the bone marrow environment, homing to the spleen was evaluated. Consistent with that seen in marrow, I demonstrate that there was no difference in $CD34^+$ cell homing to the spleen when $CD8^+$ or $CD4^+$ cells were admixed with $CD34^+$ cells. My finding on homing to the spleen also contrast with the observations of Adams and co-workers who demonstrated increased $CD34^+$ homing to the spleen when $CD8^+$ cells were added (Adams *et al*, 2003).

In summary, I have demonstrated that undifferentiated MAPCs generated from normal BM donors showed the ability to differentiate into endothelial-like cells that have *in vitro* functional characteristics that are indistinguishable from those of mature endothelial cells. I have also demonstrated that adult MPB $CD34^+$ cells co-transplanted with MSCs or MAPCs and $CD4^+$ or $CD8^+$ T cells showed no significant difference in homing levels to the BM in the NOD/SCID mice.

Chapter 7: Conclusions

7.1 CONCLUSIONS

The main question that this thesis has attempted to address is whether the reduced engraftment of cytokine activated adult human $CD34^+$ cells is due to alterations in homing behaviour and the basis for such alterations. The data presented go some way towards answering these questions.

In Chapter 3, I showed, that MPB $CD34^+$ cells cultured in serum free medium with cytokines have reduced engraftment *in vivo*, which is evident by 48 hours of culture. Ex-vivo culture had a striking effect on the homing behaviour of $CD34^+$ cells, resulting in a marked fall in homing to the BM, with a concomitant increase in cells localising to the lungs. I tested the hypothesis that such alterations in homing behaviour are due to cell cycle progression, using two different approaches in Chapter 4. In the first approach, sorted G_0/G_1 cells showed slightly higher homing to the BM compared with sorted cells in $S/G_2/M$, this did not reach significance, and remained significantly lower than the levels seen with fresh cells. Importantly, sorted cells in different phases of the cell cycle did not show differences in engraftment. In the second approach, cells induced to accumulate at the G_1/S border by treatment with aphidicolin did not show increased homing to the BM, compared with control cultured cells.

Cytokine activated $CD34^+$ cells expressing high levels of Fas may undergo apoptosis *in vivo* upon binding membrane FasL on vascular endothelium. In Chapter 5 I demonstrated that Fas levels on $CD34^+$ cells rise rapidly (by 48-72 hours) following cytokine exposure. My attempts to block Fas engagement on $CD34^+$ cells using either soluble FasL, or blocking anti-Fas mAb did not however lead to restoration of homing ability in cultured $CD34^+$ cells. Other strategies to restore the engraftment potential of cytokine-activated HSPC included altering culture conditions. Omitting IL-3 or addition of SDF-1 and retronectin from my cultures had no effect on the reduced BM homing of cultured cells. Finally, the data in Chapter 6, showed that adult MPB $CD34^+$ cells co-transplanted with accessory cells (such as MSCs, MAPCs or heterologous T cells) have no significant difference in homing levels to the BM compared with $CD34^+$ cells injected alone.

Thus, in adult $CD34^+$ cells cytokine induced loss of homing to the BM is a phenomenon that occurs rapidly and does not appear to relate to cell cycle progression. It is also not easily explained by surface CXCR4 or Fas receptor levels, or adhesion and migration behaviour *in vitro*. I suggest, based on data presented in this thesis, an alternative explanation which takes into account the tissue distribution of infused $CD34^+$ cells. Although migration within the BM and subsequent adhesion to endosteal niches are important steps in homing, it is the initial encounter with vascular endothelium that dictates the tissue destination of circulating $CD34^+$ cells. Previous work by Yong *et al.*, (1998) has shown that freshly isolated cord and adult $CD34^+$ cells do not migrate across endothelium unless activated by cytokines (Yong *et al*, 1998), a finding subsequently confirmed by other investigators (Voermans *et al*, 1999). This effect of cytokine stimulation on transendothelial migration applies to both random and SDF-1 directed migration (Yong *et al*, 1998). Thus, when infused *in vivo*, cytokine stimulated cells have a greater propensity to migrate out of the vasculature. This could lead to the inappropriate egress of transplanted cells into non-haemopoietic tissue. Unstimulated cells, on the other hand, have low migratory potential, only undergoing transendothelial migration when stimulated by appropriate cytokines within the BM micro-vasculature. This would be a mechanism for the selective egress of $CD34^+$ cells into the BM. Impaired BM homing and hence engraftment that occurs as a result of ex-vivo cytokine exposure thus relates to changes in the tissue pattern of homing. The work from this thesis suggest that cytokine stimulation increases $CD34^+$ cell localisation to the lungs while reducing homing to the BM and would support such a hypothesis. The increased apoptotic tendency of cytokine activated cells may lead to the loss of any cells that had migrated into non-haemopoietic tissue, thus contributing to the loss of engraftment potential in cultured cells.

Future directions

One issue raised by these studies is the adequacy, or appropriateness of the intravenous transplantation model for addressing the *in vivo* mechanisms of homing and engraftment. Conventionally HSPC repopulation assays are

based on intravenous injection, a complex process that requires circulation through blood, recognition of endothelium and extravasation through bone marrow vasculature, and migration to a supportive microenvironment. Cells travel into the right atrium, ventricle, and lungs in which most of the cells are trapped, then into the systemic circulation and lodge in organs according to organ blood flow, therefore, only a small fraction of injected cells initially arrive in the BM. Hence this system may detect a low homing efficiency with only 3-6% of the infused cells homing to the BM (van Hennik *et al*, 1999;Cashman & Eaves, 2000) and some classes of HSPCs may remain undetected.

A new sensitive assay of assessing stem cell potential is the intra-BM transplantation technique (Kushida *et al*, 2001;Yahata *et al*, 2003;Mazurier *et al*, 2003). The assay involves directly injecting cells into the bone marrow cavity, thus excluding the stem cell homing interference (e.g. stem cell trapping in lung and/or liver, transendothelial migration step). Therefore it may be possible to avoid the cells distributing to organs with rich vascular beds (e.g. liver and lungs) after intravenous administration. This approach might yield a more immediate and successful outcome than manipulations to enhance homing to bone marrow, especially when cultured cells are used for transplantation. It will also enable us to address the question: is it migration into the BM or the subsequent anchorage step that is altered in cytokine activated $CD34^+$ cells.

Recently another approach has been shown in a syngeneic mouse model to improve the localisation of primitive haemopoietic cells to the BM but not engraftment (Chabner *et al*, 2004). This approach involves the injection of donor primitive haemopoietic cells into the femoral artery through an intra-arterial catheter. Intra-arterial delivery of BM cells resulted in a 10-fold excess of cells homing (one hour following injection) in the ipsilateral BM compared with the contralateral BM and a 47-fold excess compared with intravenously injected cells (Chabner *et al*, 2004). This suggests that local arterial injection markedly enhanced the delivery of primitive cells to the local haemopoietic site. In contrast to the results from the homing assays, Chabner and co-

workers saw similar levels of engraftment of CD45.1 cells (in both the Ipsilateral and contralateral BM) irrespective of their route of injection (intravenous vs intra-arterial). Therefore, unlike direct injections into the marrow cavity, concentrated stem cell delivery by vascular route does not improve engraftment in the BM, suggesting that in this murine model, the homing of intravenously injected stem cells is efficient to the BM.

As mentioned earlier cytokine activated $CD34^+$ cells may have a problem in securing anchorage to appropriate HSPC niches. Two alternative ways to look at the fate of transplanted cells at the single cell level are *in situ* fixation by perfusion (Nilsson *et al*, 2001) and through an optical window surgically implanted on the mouse femur (Askenasy & Farkas, 2002;Askenasy *et al*, 2002). Both these approaches may also enable us to address the question: is it migration into the BM or the subsequent anchorage step that is altered in cytokine activated $CD34^+$ cells? Nilsson and co-workers investigated the spatial distribution of CFSE labelled HPCs within the femoral BM of nonablated recipients *in situ* fixation by perfusion. Candidate stem cells demonstrated selective redistribution and were significantly enriched within the endosteal region, whereas mature terminally differentiated and lineage-committed cells selectively redistributed away from the endosteal region and were predominantly in the central marrow region (Nilsson *et al*, 2001). Thus, the failure of the 'homing' process may be a failure to migrate into appropriate niches and/or secure anchorage within these niches.

This work also raises the question: Why have studies on stem cell homing produced conflicting observations? These differences may be due to methodological differences for example surrogate vs direct homing assays. Generally two techniques have been used to quantify homing. The first is one in which whole unseparated marrow is infused and a surrogate assay (e.g. functional CFC assay) is used as marker for stem cells (Frenette *et al*, 1998;Szilvassy *et al*, 1999;Oostendorp *et al*, 1999). However, this approach raises concerns for the validity of the surrogate assay and the possibility that the biological phenotype of the cell may change after engraftment. In the second, purified stem cells are labelled (for e.g. with fluorescent dye PKH26)

to enable them to be tracked after infusion (Hendrikx *et al*, 1996;Lanzkron *et al*, 1999;Cui *et al*, 1999;Szilvassy *et al*, 2001b). This second approach is difficult because of the relatively large number of purified stem cells needed. Other reasons for conflicting observations include *in vitro* vs *in vivo* experiments and differences in cell source such as UCB vs MPB $CD34^+$ cells. When foetal liver cells were used for homing studies, their homing to adult recipients was at least 10-fold lower than homing to adult BM cells (Szilvassy *et al*, 2001a). These findings were confirmed by Srour *et al.,* (2001), for foetal liver cells irrespective of their gestation period and for cord blood (Srour *et al*, 2001). In contrast to these data, however, van Hennik *et al.,* (1999) did not find that foetal liver cells or human CB cells had inferior homing in NOD/SCID mice (van Hennik *et al*, 1999). Several of the *in vitro* data do not appear to predict the *in vivo* situation of homing. My findings on *in vivo* homing of cytokine stimulated $CD34^+$ cells also contrast with observations that the *in vitro* adhesive function of these cells alters with cell cycle. Yong *et al.,* (2002), and others, have found *in vitro* that cultured $CD34^+$ cells exhibit increased migration and reduced adhesion whilst in G_0/G_1 phase of the cell cycle compared to cells in $S/G_2/M$ (Yong *et al*, 2002b;Huygen *et al*, 2002). These observations highlight the fact that the regulation of HSC homing to the BM is a complex process and involves interplay between adhesion molecules, chemokines, and differential shear stress in the vascular compartment. Finally, to what extent results with NOD/SCID or NOD/SCID β2m mice can be directly extrapolated to firm conclusions about human cell homing is currently being explored.

Bone marrow homing, apart from its biological significance, is of practical importance because it can influence the outcome of engraftment in clinical transplantation. However, the development of strategies to improve the efficiency of *ex vivo* expanded stem cell grafts will depend heavily on a full understanding of the molecular mechanisms that govern the homing of haemopoietic stem cells.

PUBLICATION ARISING FROM WORK PRESENTED IN THIS THESIS

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Appendix I

List of materials, plastics, antibodies, reagents (including compositions), equipment and suppliers

ITEM	SIZE	SUPPLIER	CAT NO
Plastics and tissue culture materials			
96 well flat bottomed tissue culture plate	96 well	Nunc	167008
Bijou polypropylene vials	7ml	Sterilin	129B
Filter	0.22µm	Sartorius	16532 GU
Fine tip pastettes	2ml	Alpha Labs	LW4070
Kwill/straw	5"	Avon Medicals	E910
Lab-Tek chamber slides w/cover	1 well	Nalge Nunc Inc	177410
Microscope slides		BDH	406/0181/04
Pipette tips	200µl	Sarstedt	70.760.002
	1000µl		70.762
Sterile glass pipette	1 ml	Volac	D804/PS
Sterile plastic pipettes	5ml	Sterilin	40305
	10mls		47310
	25mls		40125
Sterile polypropylene universal	50ml	Greiner	210261
Sterile polypropylene universal	15ml	Falcon	352097
Sterile polystyrene universal	30ml	Sterilin	128A
Tissue culture flasks	$T25^2$	Nunc	153732
	$T75^2$		163371
Tissue culture flasks (HUVECs)	$T25^2$	Falcon	353014
	$T75^2$		3530324
Tissue culture plates	6 well	Costar	3516
	12 well		3513
	24 well		3524
	48 well		3548
Equipment for cell isolation			
Cannulae plugs		Vygon	9888
Capped plastic test tubes for flow cytometry		Becton Dickinson Falcon Division	352054
Cell strainer	40µM	BD	352340
Click lock injection site plug		Vygon	5811.01
Cryogenic vials	1.8ml	Nalge	5000-0020
Disposable cytofunnels		Shandon	5991040
Disposable syringes	50ml	Becton Dickinson	300866
Disposable syringes	1ml	Terumo	BS-01T
	2ml		SS-02S
	5ml		BS-05S04
	10ml		SS-10ES
	20ml		BS-20ES
Intravenous cannulae (14 gauge) sterile		Abbotts	4535-14
Microfuge eppendorf tubes	0.5ml	Sarstedt	72.692.005
	1ml		
Parafilm	2" x 250 ft	BDH	PM-992
Sterile needles	26g Microlance 3	BD	303800

ITEM	SIZE	SUPPLIER	CAT NO
Sterile needles	0.8 x 40mm	Neolus	NN-2138R
Sterile surgical swabs and towels (10x10)		Smith and Nephew	90623255
Surgical blade		Swann-Mortan	0507
General cell culture reagents			
20% Human Albumin Grifols	50ml	ALPHA	PL12930/0001
Bovine serum albumin	100g	Sigma	A-4503
$CaCl_2$			
Collagenase A from *Closridium histolyticum*		Boerhinger Mannheim	
D(+)-Glucose	500g	BDH	101174Y
Dexamethasone		Sigma	D2915
Dimethyl Sulphoxide (DMSO)	10 ml ampoule (Tissue culture grade)	Sigma	D2650
DMEM low glucose	500ml	Gibco BRL	31885-023
EDTA	500g	BDH	104284C
Endothelial cell growth supplement (ECGS)	15mg	Sigma	E-2759
Fibronectin- from human plasma	5mg	Sigma	F-2006
Ficoll-Paque	500ml	Pharmacia Biotech	17-0840-03
Foetal bovine serum (FBS) for MAPCs; ES screened US origin-heat inactivated at 56°C for 30 min	500ml	Hyclone	
Foetal calf serum- heat inactivated at 56°C for 30 min	500ml	PAA Labs	A15-773
Hank's Balanced Salt Solution (HBSS)	500ml	Gibco BRL	14170
Heparin (monoparin)	1,000U/ml	CP Pharmaceuticals Ltd., Wrexham, UK	BN95013559
HEPES	50g	Sigma	H7523
Iscove's (IMDM) with Glutamax-1	500ml	Gibco BRL	31980
ITS	100x	Sigma	I3146
LA-BSA	100mg/ml	Sigma	L9530
L-Ascorbic acid 2 phosphate		Sigma	A8960
Matrigel	10ml	Becton Dickinson	354234
MCDB-201		Sigma	M6770
Methocult	80ml	Stem cell Technologies Inc (Dist by Metachem. Diagnostics Ltd in UK)	H4230
Mouse serum	1ml	Dako	X0910
NaCl	500g	BDH	102414J
$NaHCO_3$	500g	BDH	102474V
NH_4Cl	500g	BDH	100173D
Paraformaldehyde		Sigma	P6148
Penicillin / Streptomycin	5000U/ 5mg	Sigma	P4458
Phosphate Buffered Saline	with Ca^{++} or Mg^{++}	Gibco BRL	14040-091
Phosphate Buffered Saline	without Ca^{++} or Mg^{++}	Gibco BRL	14190-094
RetroNectin	0.5mg	Takara Biomedicals	T100A
RPMI 1640 with L-glutamine	500ml	Gibco BRL	21875-034
Sodium azide	100g	Sigma	S-2002
Trypsin/EDTA	100ml	Sigma	T4299

ITEM	SIZE	SUPPLIER	CAT NO
Water for irrigation BP	10ml	Braun Hospital pharmacy	3627608
X-VIVO 10	1000ml	Biowhittaker	04-380Q
Recombinant human growth factors and chemokines:			
EGF	200µg	Sigma	E9644
Erythropoietin	1000U/ml	Boehringer Mannheim	PL15722
Fas Ligand Plus recombinant protein	5µg	Oncogene Research products	PF092
Fms-like tyrosine kinase 3 ligand (Flt3L)	10µg/ml	Peprotechec	300-19
G-CSF	250µg/ml	Chugai Pharma	
granulocyte/monocyte - colony stimulating factor (GM-CSF)	250µg/ml	Hoechst	GF-409-61
IL-3	300µg/ml	Sandoz	(clinical trial vial)
Interleukin-6 (IL-6)	20µg/ml	Peprotechec	200-06
PDGF-BB	10µg	R&D sysytems	220-BB
Soluble Fas Ligand (sFasL)	10µg	Alexis Corporation Ltd	522-001-C010
Stem Cell Factor (SCF)	10µg/ml	Peprotechec	300-07
Stromal cell derived factor-1a (SDF-1a)	10µg	Peprotechec	300-28A
Thrombopoietin (TPO)	10µg	Peprotechec	300-18
Vascular endothelial cell growth factor (VEGF)	10µg	Peprotechec	100-02
Immunohistochemistry			
100% alcohol		BDH	
DAB tablets	10mg	Bio-Stat Ltd Ken-En-Tec AS Denmark	896102
Formalsaline		Sigma	
Formic acid	500ml	Fisons	F/1900/PB08
Hydrogen peroxide (H_2O_2)		Sigma	95294
Methanol	1L	BDH	10158AF
Sodium chloride		Sigma	
Sodium citrate		Sigma	
Styrolite mounting medium	500ml	BDH	361704Y
Tris		Sigma	
Xylene	1L	BDH	120932D

ITEM	SIZE	SUPPLIER	CAT NO
Antibodies for flow cytometry			
anti-CD34 PE		BD Biosciences	345802
Anti-CD34 FITC		BD Biosciences	345801
Anti-CD38 PE		BD Biosciences	345806
Anti-CD45 FITC		BD Biosciences	345808
Anti-CD45 PerCP		BD Biosciences	345809
Anti-CD3 FITC		BD Biosciences	345763
Anti-CD2 PE		BD Biosciences	347597
Anti-CD19 PE		BD Biosciences	345777
Anti-CXCR4 PE	2ml	BD Pharmingen	555974
Anti-CD95 PE	2ml	BD Pharmingen	555574
Anti-CD31 PE		BD Biosciences	340297
Anti-CD44 FITC	2ml	BD Pharmingen	555478
Anti-CD49b FITC	2ml	BD Pharmingen	555498
Anti-VLA4 (CD49d) PE		BD Biosciences	340296
Anti-VLA5 (CD49e) PE	2ml	BD Pharmingen	33225X
Anti-CD11a FITC		BD Biosciences	347983
Anti-IgG1 PE isotype control		BD Biosciences	349043
Anti-IgM FITC isotype control		BD Biosciences	345815
Anti-IgG1 PerCP isotype control		BD Biosciences	345817
Anti- IgG2a mouse PE isotype control		BD Pharmingen	5555574
SH2 FITC		Stem Cell Technologies	
SH4 FITC		Stem Cell Technologies	
HLA-DR APC		BD Pharmingen	
HLA-ABC FITC		BD Pharmingen	
Anti-CD13 FITC	1ml	Dako	F0831
Anti-CD38 FITC	1ml	Dako	F7101
Anti-IgG1 FITC isotype control	1ml	Dako	X0927
Anti-CD8 FITC/CD4 RD1/CD3 PC5	0.5ml	Beckman Coulter	PN6607053
Anti-mouse IgG1 FITC/RD1/PC5 isotype control	0.5ml	Beckman Coulter	PN660754
Antibodies for immunohistochemistry			
Anti-CD45RB	1ml	Dako	M0833
MOM Immunodetection kit		Vector Labs	PK2200
Antibodies for immunocytochemistry			
Anti-Von Willebrand factor	1ml	Dako	M0616
Rabbit anti-mouse FITC	2ml	Dako	F0313
Blocking antibodies			
Anti-Fas	100μg in 100μl	MBL Ltd	MD113
Inhibitors and enzymes			
RNAase		Sigma	R5503
Aphidicolin from *Nigrospora sphaerica*		Sigma	A0781

ITEM	SIZE	SUPPLIER	CAT NO
Dyes and stains			
0.4% Trypan Blue	100ml	Sigma	T8154
Annexin V FLUOS	500µl	Roche	1828681
DiI-Ac-LDL	200µg/ml	tebu-bio	4003
Hoechst 33342	100mg	Molecular Probes	H-1399
PKH26 staining kit		Sigma	PKH26-GL
Propidium Iodide		Sigma	P4170
Pyronin Y	1g	Sigma	P9172
Vectorshield mountant	10ml	Vector Labs	H-1000
Positive cell selection			
CliniMACS buffer		Miltenyi Biotec	
Human CD34 Microbeads		Miltenyi Biotec	
Human CD4 Microbeads	2ml	Miltenyi Biotec	130-045-101
Human CD8 Microbeads	2ml	Miltenyi Biotec	130-045-201
Pre-separation filters 30µM		Miltenyi Biotec	130-041-407
VarioMACS LS column		Miltenyi Biotec	130-041-306
Negative cell selection			
Human CD45 Microbeads	2ml	Miltenyi Biotec	130-045-801
Human Glycophorin A Microbeads	2ml	Miltenyi Biotec	130-050-501
LD column		Miltenyi Biotec	130-042-901

Equipment

Equipment	*Model and Company*
$C0_2$ Incubator	Galaxy R, Scientific Lab Supplies Ltd
Centrifuges	Centaur 2, MSE MS3000, MSE
Class II Microbiological safety cabinet	Walker safety cabinets Ltd, UK
Flow cytometers Epics Elite FACS vantage (cell sort)	Beckman Coulter, UK
Inverted microscopes	211102 Olympus, Japan Zeiss Stemi 2000-C
Irradiator	Gammacell CIS IBL 437
Light microscope	BH-2 Olympus, Tokyo
Phase contrast microscope	Phase contrast 2 18813 Nikon, Japan

Buffers and solutions

Citrate buffer:

Sodium citrate	8.82g

Made up to 3 litre with dd H_2O and pH adjusted to 6

Freeze mix solutions:

$CD34^+$ cells:

PBS w/o Ca_2+ Mg_2+	9ml
DMSO (v/v)	3ml
HSA 20% (v/v)	8ml

Human Umbilical Vein Endothelial Cells:

Iscoves MDM	6ml
FCS	2ml
DMSO	2ml

Made up fresh and chilled at 4°C for 30 minutes just before use.

Hoechst buffer:

D Glucose	200mg
HEPES	953mg
FCS	20ml
HBSS	180ml

pH adjusted to 7.3 and filtered through a 0.22µM filter and stored at 4°C

PI cell cycle staining solution:

Propidium iodide 2mg/ml in ddH_20	200µl
RNase 10mg/ml in 10mM Tris (pH 7.5) 15mM NaCl	500µl
PBS w/o Ca_2+ Mg_2+	10ml

Pass through a 0.2µM filter and store at 4°C

RBC lysis buffer:

NH4Cl	8.3g
NaHCO3	1.68g
EDTA	0.4g

Made up to 1000ml with ddH_2O, filtered solution through a 0.22µM filter and stored in 50ml aliquots at –20°C

RBC lysis rescue buffer:

BSA 5% (w/v)	10ml
Glucose 10% (w/v)	5ml
HBSS	35ml

Filtered through a 0.22µM filter and stored at 4°C

Staining buffer:

PBS w/o Ca_2+ Mg_2+	35ml
BSA 5% (w/v)	10ml
Sodium azide 1% (w/v)	5ml

Filtered through a 0.22μM filter and stored at 4°C

Tris buffered saline (TBS):

Sodium chloride	80g
TRIS	6.05g

Made up to 10 litre with dd H_2O and pH adjusted to 7.6

Wash buffer:

PBS w/o Ca_2+ Mg_2+	40ml
BSA 5% (w/v)	10ml

Filtered through a 0.22μM filter and stored at 4°C

Culture media

HUVEC complete culture medium:

Iscove's MDM	16ml
FCS	4ml
Heparin 1000U/ml	400μl
ECGS 1mg/ml	332μl

Filtered through a 0.22μM filter and stored at 4°C

MAPC expansion medium:

DMEM low glucose	12ml
MCDB-201	8ml
ITS x100	200μl
LA-BSA 100mg/ml	100μl
Ascorbic acid-2-phosphate 10mM	200μl
FBS (Hyclone)	400μl
Dexamethasone 1μM	1ml
PDGF-BB 10μg/ml	20μl
EGF 100μg/ml	2μl

Filtered through a 0.22μM filter and stored at 4°C.

Stock solutions

Aphidicolin reconstitution:

Aphidicolin	1mg

Reconstituted in 1ml DMSO and stored at –20°C (stable for 6 weeks).

L-ascorbic acid 2 phosphate 1M solution:

Ascorbic acid-2-phosphate 2.561g
Made up to 10 ml with dd H_2O and filtered and stored at 4°C

BM, spleen, lungs and liver collection media:

RPMI 1640	45ml
FCS	5ml
Heparin 1000U/ml	1ml

5% BSA solution:

BSA 2.5g
Made up to 50ml with HBSS and filtered through a 0.22μM filter and stores at 4°C

Dexamethasone 1mM solution:

Dexamethasone 3.92g
Made up to 10ml with dd H_2O and filtered and stored at 4°C

EGF human recombinant solution:

EGF 200μg
Reconstituted in 2ml of 10mM acetic acid+0.1% HAS and 100μl aliquots stored at –20°C

FasL Plus solution (recombinant protein)

FasL Plus 5μg
Dissolved in 50μl of sterile water and 10μl aliquots frozen at –20°C (stable for 1 year).
Further dilutions were made with medium containing 5% FCS.

sFas Ligand (soluble, human recombinant):

Fas Ligand 10μg
Dissolved in 100μl of ddH_2O and aliquots stored at –20°C. Further dilutions were made with medium containing 5% FCS.

Fibronectin solution:

Fibronectin	5mg
Water for irrigation	2ml
HBSS	8ml

Aliqouts of 2 ml in autoclaved glass bottles were stored at –20°C. For use an aliquot was thawed and made up to 8ml with HBSS and plastic coated appropriately.

10% Glucose solution:

Glucose 5g
Made up to 50ml with HBSS and filtered through a 0.22μM filter and stored at 4°C

MCDB-201 solution:

MCDB 201 M6770	Powdered media (1 bottle)

Made up to 900 ml with dd H_2O and pH adjusted to 7.3 (with the use of 1N HCl or 1N NaOH). Add additional dd H_2O to bring the solution to final volume (1 Litre) and sterilised immediately by filtration using a membrane with a porosity of 0.22 microns. Medium was dispensed into sterile containers (T80 tissue culture flasks, Nalge Nunc) and stored at 4°C.

PDGF-BB human recombinant solution:

PDGF-BB	10µg

Reconstituted in 1ml of 4mM HCl +0.1% HSA and 50µl aliquots stored –20°C.

Retronectin solution:

Retronectin	0.5mg
dd H_2O	0.5ml
PBS w/o Ca_2+ Mg_2+	4.5ml

Filtered through a 0.5µm filter and 200µl aliquots stored at –20°C.

RNAase stock solution:

10mg/ml RNAase in 10mM Tris (pH 7.5)

15mM NaCl

Filter solution and heat to 100°C for 15 minutes (to destroy any contaminating DNAase). Aliquot and store at –20°C.

1% Sodium Azide solution:

Sodium azide	250mg

Made up to 25ml with dd H_2O and filtered through a 0.22µM filter and stored at 4°C

Propidium iodide stock solution:

2mg/ml in distilled water