Mechanisms underlying cytokine-induced changes in homing and engraftment of human haemopoietic stem and progenitor cells

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Declaration

I, Konstantina Kallinikou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:…………………………

Date:…………………………
Abstract

The reduced engraftment potential of cytokine cultured haemopoietic stem/progenitor cells from adult mobilised peripheral blood has been associated with their defective homing to bone marrow niches. In this work, using established in vivo systems and a novel ex vivo model, an additional cytokine-induced attachment defect is described that reduces the retention of these cells in the bone marrow, post-transplantation. This defect was found to be related to specific niche ligands and was not caused by downregulation of their respective receptors on the expanded cells. CD26 is a protease that cleaves SDF-1 abrogating its chemotactic effect. CD26 inhibition on the transplanted cells was not sufficient to reverse the engraftment defect, although infusion of the inhibitor in immunodeficient animals, together with ex vivo treated cells, significantly increased engraftment. Finally, mobilised peripheral blood stem/progenitor cells were found to express neuroreceptors and their expression was altered after exposure to cytokines. Epinephrine pre-treatment of these cells rescued their adhesion to specific niche ligands, increased their short-term homing and improved their long-term engraftment in immunodeficient animals.
Acknowledgments

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<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BFU-E</td>
<td>&quot;Burst&quot;-Forming Unit for Erythroid cells</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CB</td>
<td>Cord Blood</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation (such as CD34 etc.)</td>
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<td>CFC</td>
<td>Colony-Forming Cell</td>
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<tr>
<td>CFU-GM</td>
<td>Colony-Forming Unit for Granulo-Monocytic cells</td>
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<td>CMP</td>
<td>Common Myeloid Progenitor</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>Dimethyl sulfoxide</td>
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<td>ECM</td>
<td>Extracellular Matrix (proteins)</td>
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<td>EDTA</td>
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<td>Fluorescence activated cell sorter</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>LFA-1, LFA-2</td>
<td>Leukocyte-Activating Factor-1,2</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin$, Sca$, c-Kit$^*$ (cell population)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long-Term Colony Initiating Cell</td>
</tr>
<tr>
<td>LTRC</td>
<td>Long-Term Repopulating Cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MPB</td>
<td>Mobilised Peripheral Blood</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer (cells)</td>
</tr>
<tr>
<td>NS</td>
<td>Non-Significant</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic/severe combined Immunodeficient</td>
</tr>
<tr>
<td>o/n</td>
<td>overnight</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral Blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Media</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor (c-kit ligand)</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley (rat breed)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal Derived Factor-1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>SP</td>
<td>Side Population</td>
</tr>
<tr>
<td>TC</td>
<td>Tissue Culture</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin (c-mpl ligand)</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very-Late Antigen-4 (as with VLA-5)</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cells</td>
</tr>
<tr>
<td>β2m/NOD/SCID</td>
<td>β2 microglobulin/Non-obese diabetic/severe combined Immunodeficient</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Haemopoiesis

Haemopoiesis (from the Greek haema- blood and poiesis- production) is the formation of all the cellular components of blood. It is a particularly well-coordinated process, as a very high level of cell turnover is required to cover the normal daily needs of the organism, which are further increased in cases of illness or trauma. Erythrocytes survive for a few months, granulocytes for a few hours and overall, normal haemopoiesis involves the daily production of $2 \times 10^{11}$ erythrocytes, $1 \times 10^{10}$ granulocytes and $4 \times 10^{11}$ platelets as well as lymphocytes and monocytes (Tavassoli, 1980). The vast majority of these cells is generated by a comparatively small population (1-10x10$^6$ Moore, 1996) of multipotent and more differentiated, oligopotent progenitors, the haemopoietic stem and progenitor cells (HSPC), which are morphologically similar to small or medium-sized lymphocytes.

1.2 Characterisation of haemopoietic stem and progenitor cells

The primary haemopoietic stem cells (HSC) give rise to the multipotent common lymphoid and myeloid progenitors (CLP and CMP). The CLP generate the B, T and NK cells whereas the CMP produce erythrocytes, granulocytes, monocytes and megakaryocytes (the last of which release platelets) (Kondo et al, 1997; Akashi et al, 2000) (Figure 1.1). The haemopoietic stem cells are rare within the adult organism, with a frequency of approximately 1 in 20x10$^6$ nucleated cells, in the bone marrow (BM). HSC are able to divide producing identical stem cells (self-renewal) as well as more differentiated, daughter cells which are restricted in regards to their developmental potential (Spangrude et al, 1988). These primary progenitor cells are committed to produce cells of discrete lineages. The type of
Figure 1.1: Schematic representation of haemopoiesis and haemopoietic assays (adapted from thesis of F. Ahmed, UCL, 2004)
progenitors produced, as well as the downstream differentiated lineages depend on a variety of stochastic or external factors, such as the cytokines present in the microenvironment of the cells. A great range of protocols have been utilised to select or enrich for HSC from mixed mononuclear cell populations. These include density centrifugation, sensitivity to cell cycle-active cytotoxic agents (Szilvassy & Cory, 1993; Orschell-Traycoff et al, 2000) dye efflux properties (Goodell et al, 1996) and expression of surface antigens (Wilson et al, 2007)

1.2.1 Cell surface markers

Identification of a single or a combination of surface antigens for HSC would significantly aid characterisation and isolation of this small population. So far no single phenotypic characteristic has been described that specifically defines either human or murine HSC. Through a variety of functional assays, however, a number of receptor combinations have been proposed to enrich for HSC, especially in the murine system, such as the LSK and SLAM codes described below.

Primarily, HSC are devoid of antigens linked to specific lineages such as CD45R/B220, CD3, CD4, CD8, which are expressed on terminally differentiated lymphocytes, TER-119/molecule associated with cell surface Glycophorin A, which identifies erythroid cells and CD11b/Mac-1, Ly-6G/Gr-1 expressed on myeloid cells. A number of HSC enrichment protocols, therefore, involve negative selection to exclude all the cells expressing the antigens associated with lineage commitment.

1.2.1.1 CD34

CD34 is a cell-surface sialomucin (a sialic acid transmembrane glycoprotein) which has been established as the main marker for the enrichment of haemopoietic stem cells and is thus widely used for the isolation and
purification of such cells on the bench and in clinic. The function of this receptor for HSC is largely unknown, although it has been associated with enhancement of proliferation, inhibition of differentiation and regulation of HSC trafficking. The last function is related to its role as an inhibitor of adhesion and enhancer of transmigration (Healy et al., 1995; Lanza et al., 2001; Nielsen & McNagny, 2009). CD34 is expressed on populations of primitive stem and progenitor cells and its expression decreases as the cells differentiate (Civin et al., 1984; Andrews et al., 1989; Krause et al., 1996). The CMP and its immediate progeny (the granulocyte/macrophage and megakaryocyte/erythrocyte progenitors), for example, express CD34, whereas the terminally differentiated cells (such as granulocytes & macrophages) do not (Manz et al., 2002).

CD34 was first identified from a human acute myeloid leukemia cell line, against which the My10 antibody was raised (Civin et al., 1984). Other monoclonal antibodies specific for CD34 include the clones HPCA2 (8G12) and QBEnd10, the latter of which is used by the commercially available selection system produced by Miltenyi Biotec and used for clinical selection of HSPC (Watts et al., 2002). Although CD34 is expressed by the majority of HSPC (as identified by functional assays) not all CD34+ cells are clonogenic. For example, a purified, CD34 positive cell population from peripheral blood (PB) has a progenitor frequency of 19.42% (2.67%-56.74%), (median and range from 118 donors; personal communication from Stuart Ings, Wolfson Cellular Therapy Unit, University College London Hospitals). Furthermore, CD34 is also expressed in various other cell types including most of the vascular endothelial cells (Fina et al., 1990) and some fibroblasts (Brown et al., 1991). Moreover, not all HSPC are positive for CD34. In fact the most primitive, dormant HSC have been reported to be CD34lo- (Wilson et al., 2007).
1.2.1.2 Other HSC markers

Additional markers of HSC are listed in Table 1.1 (adapted from Szilvassy, 2003). The majority of these markers are associated with differentiated cells, such as CD38, which is expressed on WBC lineages but not on HSC (Peled et al., 1999b). For this reason most listed markers have low or negative expression on HSC. AC133, KDR, CD150, CD201, Tie2 and Sca-1 (for mice) are some of the few positive indicators of HSC.

1.2.1.2.1 AC133 and KDR

AC133 (CD133) is a 5-transmembrane glycoprotein, of unknown function so far, which is expressed on human HSC, as well as endothelial progenitors, glial stem cells and a number of other cell types. Kinase insert Domain Receptor (KDR) is also known as vascular endothelial growth factor receptor 2 (VEGFR-2) or Foetal liver kinase 1 (Flk1). KDR has been found on endothelial cells (Asahara et al., 1997), while its mRNA is present in megakaryocytes. Expression of KDR has also been detected on human haemopoietic cells (Katoh et al., 1995) and has been associated with the most undifferentiated, pluripotent fraction of this population (Ziegler et al., 1999). In humans, the subpopulation that combines these three receptors (CD34, CD133 and KDR) has been shown to contain primitive haemopoietic progenitors (Case et al., 2007).

1.2.1.2.2 CD150

CD150 is another antigen that has been positively associated with HSPC. This antigen is normally expressed on T-cells, thymocytes, B-cells, dendritic cells and monocytes. Weksberg and colleagues have reported that murine HSPC, both positive and negative for CD150, have long-term haemopoietic activity (Weksberg et al., 2008), although expression of this marker was not found in human HSPC (Sintes et al., 2008).
<table>
<thead>
<tr>
<th>Stem cells</th>
<th>Progenitor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
</tr>
<tr>
<td>CD34^+ and CD38^-</td>
<td>CD34^+</td>
</tr>
<tr>
<td>Thy-1^lo</td>
<td>Thy-1^-</td>
</tr>
<tr>
<td>c-kit^lo</td>
<td>c-kit^+</td>
</tr>
<tr>
<td>HLA-DR^+/lo</td>
<td>HLA-DR^+</td>
</tr>
<tr>
<td>CD45RA^-/lo</td>
<td></td>
</tr>
<tr>
<td>CD71^-/lo</td>
<td></td>
</tr>
<tr>
<td>AC133^+</td>
<td>Rh-123^hi</td>
</tr>
<tr>
<td>Rh-123^-/lo</td>
<td>HO^-/lo</td>
</tr>
<tr>
<td>HO^-</td>
<td>KDR^-/lo</td>
</tr>
<tr>
<td>KDR^+ (Flk1^+)</td>
<td></td>
</tr>
<tr>
<td><strong>Adult Mouse BM</strong></td>
<td></td>
</tr>
<tr>
<td>Sca-1^-/+</td>
<td>Sca-1^-/+</td>
</tr>
<tr>
<td>CD34^-</td>
<td>CD34^-/lo</td>
</tr>
<tr>
<td>CD38^+</td>
<td>CD38^+</td>
</tr>
<tr>
<td>c-kit^-/lo</td>
<td>c-kit^-/lo</td>
</tr>
<tr>
<td>Lin^-</td>
<td>Lin^-</td>
</tr>
<tr>
<td>Thy-1^-/lo</td>
<td>Thy-1^-</td>
</tr>
<tr>
<td>HO^-/lo</td>
<td>HO^-/lo</td>
</tr>
<tr>
<td>Flk-2^-</td>
<td>Flk-2^-</td>
</tr>
</tbody>
</table>

Table 1.1: Phenotype of human and murine haemopoietic stem and progenitor cells. Adapted from (Szilvassy, 2003).
1.2.1.2.3  **CD201**

CD201 or endothelial protein C receptor (EPCR) is the receptor for activated protein C, which is a blood coagulation factor (Fukudome & Esmon, 1995). Murine EPCR\(^+\) BM cells co-express the well-established HSC markers and have been shown to have high repopulating activity *in vivo* (Balazs *et al*, 2006).

1.2.1.2.4  **Tie2**

Tie2 is receptor tyrosine kinase that binds to angiopoietin. Expression of Tie2 has been detected on endothelial cells (Dumont *et al*, 1992; Sato *et al*, 1993) as well as on murine HSPC (Iwama *et al*, 1993; Hsu *et al*, 2000). The Suda group has demonstrated that, in the murine system, Tie2 expressing HSC are quiescent and are located in close proximity to osteoblasts in the BM niches, *in vivo* (Arai *et al*, 2004, 2).

1.2.1.2.5  **Thy-1**

Thy-1 (or CD90) is an N-glycosylated, glycophasphatidylinositol-anchored cell surface protein that is expressed on mesenchymal and liver stem cells as well as haemopoietic stem cells in humans, mice, and rats. It was originally discovered as an antigen against thymocytes. In humans all Thy-1 positive cells are also CD34 positive (Stewart *et al*, 1995). Within the HSC population, low Thy-1 expression has been associated with the more primitive cells (Uchida & Weissman, 1992; Uchida *et al*, 1994).

1.2.1.2.6  **Sca-1**

Since 1988 Stem cell antigen-1 (Sca-1) has been described as a marker of multipotent HSPC in the murine system (Spangrude *et al*, 1988). It is a
phosphatidylinositol-anchored protein, and a member of the Ly-6 antigen family (Van de Rijn et al, 1989). Sca-1 may be involved in B and T cell activation and expression of it has been detected in non-haemopoietic cell populations, where it has been used to enrich for progenitor/stem cells (Welm et al, 2002). A combination of Sca-1 and c-Kit expression, with absence of lineage related markers is the standard protocol for the selection of HSPC in the murine system. The Lin− Sca-1+c-Kit+ (LSK) population, however, includes many heterogeneous cells, as only approximately 10% of these cells have repopulation ability.

The reconstitution capacity of defined sub-populations of HSC based on combined expression of specific receptors has been extensively tested in mouse models where, following lethal irradiation, single selected HSC have been transplanted, with varying levels of engraftment efficacy (summarised in Table 1.2, adapted from Wilson et al, 2007).

### 1.2.1.3 Dye efflux properties

HSPC have the ability to efflux DNA-binding dyes such as Rhodamine-123 (Uchida et al, 2003) or Hoechst 33342, via the ATP-binding cassette (ABC) family of transporter proteins expressed within the cell membrane. These cells appear as a side population (SP) when analysed by flow cytometry (Camargo et al, 2006). This is, in principle, a functional assay that allows for the separation of HSPC from the adult mouse BM (Goodell et al, 1996). This protocol effectively selects HSPC in several other species, including humans (Goodell et al, 1997).

### 1.2.2 Functional assays

Based on cell marker and SP characterisation the most descriptive combination of the HSC within the LSK population are the SLAM receptors (CD150 and CD48) and CD34 (Table 1.2).
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Frequency of reconstitution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK, CD150&lt;sup&gt;+&lt;/sup&gt;CD48&lt;sup&gt;−&lt;/sup&gt;</td>
<td>47</td>
</tr>
<tr>
<td>CD41&lt;sup&gt;−&lt;/sup&gt;CD150&lt;sup&gt;+&lt;/sup&gt;CD48&lt;sup&gt;−&lt;/sup&gt;</td>
<td>45</td>
</tr>
<tr>
<td>LSK, SP&lt;sub&gt;lo&lt;/sub&gt;, Thy1.1&lt;sub&gt;lo&lt;/sub&gt;, CD34&lt;sup&gt;−&lt;/sup&gt;, CD135&lt;sup&gt;−&lt;/sup&gt;</td>
<td>35</td>
</tr>
<tr>
<td>Lin&lt;sup&gt;neg&lt;/sup&gt;, SP, Rho&lt;sub&gt;lo&lt;/sub&gt;</td>
<td>33</td>
</tr>
<tr>
<td>LSK, CD34&lt;sup&gt;lo&lt;/sup&gt;/−</td>
<td>22</td>
</tr>
<tr>
<td>CD150&lt;sup&gt;+&lt;/sup&gt;CD48&lt;sup&gt;−&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>LSK, Thy1.1&lt;sub&gt;lo&lt;/sub&gt;</td>
<td>18</td>
</tr>
</tbody>
</table>

LSK = Lin<sup>−</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup>
SP = side population (can efflux Hoechst 33342)
SP<sub>lo</sub> = tip of SP
Rho: Rhodamine.

**Table 1.2: Single-cell BM reconstitution data according to murine surface antigens.** Murine, single-purified BM HSC were transplanted in syngeneic hosts and haemopoietic reconstitution was measured. Adapted from (Wilson et al, 2007).
This profile, which mainly applies to the murine system, has a maximal reconstitution capacity of 47% and thus does not accurately represent the HSC. For that reason, functional assays remain the most precise method of evaluating the number of progenitors or stem cells within a population.

1.2.2.1 In vitro assays

In vitro assays utilise cytokine combinations, matrixes and stromal cells to measure the proliferative potential of the seeded cells. The most established of such assays are listed here in order of differentiation of the target cells, starting from the oligopotent to the multipotent HSPC (Figure 1.1).

1.2.2.1.1 Clonogenic assays

Clonogenic assays can quantify the number of committed progenitors within a group of cells by the production of colonies after 2-3 weeks. To maintain integrity of the produced colonies, viscous media (methylcellulose) are used which are supplemented with cytokines to enhance proliferation and differentiation. The progenitors detected with this system are named after the colony type produced. Examples include the burst forming units-erythroid (BFU-E) and colony forming cells-granulocyte (CFU-G) or colony forming cells-granulocyte macrophage (CFU-GM) when the initial progenitor is bipotent and a mixed colony is produced. This assay is widely used due to the fact that it is comparatively easy and standardised through commercially available media. It is also used in clinic to assess the quality and clonogenicity of HSPC samples from donors or patients after collection or cryopreservation. The disadvantage of this assay is that the colony production is largely dependent on the specifications of the media, the serum and the cytokine combination used (reviewed in Gordon, 1993).

1.2.2.1.2 Cobblestone area-forming cell (CAFC) assays
CAFC assays utilise stromal cells and are based on the morphological (cobblestone like) appearance of groups of cells within the supporting layer (Ploemacher et al, 1989). Depending on the time of culture these assays can quantify the number progenitors (1 week) or more primitive stem cells (4 weeks), in a mixed population (Neben et al, 1993).

1.2.2.1.3 Long-term culture initiating cell (LTC-IC) assays

LTC-IC and extended LTC-IC (E-LTC-IC) assays also utilise stromal cell support (reviewed in Gordon, 1993). In the LTC-IC assay the multipotent stem cells generate clonogenic cell progeny after culture for a minimum of 5 weeks with a stromal layer (Sutherland et al, 1989). E-LTC-IC are similar to LTC-IC but arise from cultures that have been pre-cultured with cytokines (up to 10 weeks) (Hao et al, 1996). These cells have a slow, limited response to cytokine stimulation and appear to be more quiescent than LTC-IC. They have a CD34+/CD38- phenotype and are similar to the cells that reconstitute haemopoiesis in in vivo models (described below) (Bhatia et al, 1997b).

1.2.2 In vivo assays

The most definitive method of identifying a haemopoietic stem cell is to test its ability to reconstitute haemopoiesis after transplantation in a conditioned recipient. For this purpose a variety of animal models exist which support engraftment of transplanted cells. For the assessment of HSC of human origin a number of immunodeficient animal models have been developed where the host immune system does not recognise the transplanted cells as foreign and thus xenogeneic cell engraftment is permitted.

1.2.2.2.1 SCID mouse model

The first well-established animal model for human HSPC transplantation is the Severe Combined Immuno-Deficient (SCID) mouse model first described in 1983 (Bosma et al, 1983). Immunodeficiency arises due to an autosomal
recessive mutation for which the mice are homozygous. As a result they have severely impaired lymphopoiesis. They have little or no B and T cells and consequently lack both humoral and cell-mediated immunity (Bosma & Carroll, 1991). These animals fail to express rearranged antigen receptors (Lieber et al, 1988) due to defective activity of a DNA recombinase (Malynn et al, 1988). The absence of immunity in this system, however, is not absolute as a few functional B or T clones are often generated (Bosma et al, 1988; Nonoyama et al, 1993). In fact, after the age of 10-14 months, most SCID mice tend to produce functional B and/or T clones which, in the context of HSPC transplantation, leads to the requirement for high cell doses to achieve engraftment.

1.2.2.2 SCID-hu mouse model

A few years after the initial SCID model the SCID-hu mouse was described by McCune and colleagues (McCune et al, 1988). In this model implantation of small fragments of human foetal thymus and liver allowed for better support of human HSPC post transplantation (Namikawa et al, 1990). In fact, long-term retention of the human lineages was demonstrated for 5-11 months post transplantation (Namikawa et al, 1990).

1.2.2.3 NOD/SCID mouse model

Non-obese diabetic (NOD) mice spontaneously develop autoimmune insulin dependent diabetes mellitus (Kikutani & Makino, 1992; Makino et al, 1980). Crossing of NOD mice with the SCID model (NOD/SCID) was performed by two independent groups, producing the NOD/ShiLtSz-Prkdscid (NOD/Lt-scid) (Shultz et al, 1995) and NOD/ShiJic-Prkdscid (NOD/Shi-scid) mice (Koyanagi et al, 1997). These NOD/SCID strains have a less effective NK and complement function, compared to the SCID strain, as well as non-functional macrophages (Shultz et al, 1995). As a result, engraftment of human HSPC is

33
well supported and can be achieved with cell numbers 10 to 20-fold lower than those required for SCID mice.

1.2.2.2.4 $\beta_2m$/NOD/SCID mouse model

Genetic crossing of the NOD/LtSz mice, homozygous for the SCID and a $\beta_2$ microglobulin mutation, produced the NOD/SCID-$\beta_2m^{-/}$ mouse model (Christianson et al., 1997). These animals, in addition to the absence of lymphocytes and serum immunoglobulin, also lack expression of MHC class I and NK activity. Therefore, the haemopoietic system of this mouse strain can be reconstituted with as few as $8 \times 10^4$ cord blood (CB) mononuclear cells, giving a high repopulating cell frequency compared to NOD/SCID animals (Kollet et al., 2000). Moreover, short-term repopulating CD34$^+$/CD38$^+$ and CD34$^+$/CD38$^-$ populations, which do not efficiently engraft NOD/SCID mice, have been shown to reconstitute haemopoiesis in the $\beta_2m$/NOD/SCID model (Glimm et al., 2001).

1.2.2.2.5 NOG/NSG mouse models

The interleukin-2 receptor gamma (IL2R$\gamma$) chain is essential for the signaling of a variety of cytokines (such as IL-2, IL-4 and IL-7). Mutations in this gene significantly impair NK activity, (as well as B and T-cell development). Two mouse strains have been produced with IL2R$\gamma$ mutations (Cao et al., 1995; Ohbo et al., 1996). Crossing of these mice with NOD/SCID animals produced the NOD/ShiLtSz-scid/IL2R$\gamma^{null}$-(NSG) (Shultz et al., 2005) and NOD/ShiJic-scid/IL2R$\gamma^{null}$-(NOG) strains (Ito et al., 2002). Addition of the IL2R$\gamma$ mutation to a NOD/SCID background renders these mice particularly immunodeficient and subsequently very permissive of engraftment of xenogeneic HSPC (Ito et al., 2002; Shultz et al., 2005).

1.2.2.2.6 Foetal sheep model
Another animal model that bypasses the need for conditioning by irradiation or other myeloablative strategies, while supporting long-term engraftment of human HSPC, is the foetal sheep model (Zanjani et al, 1992; Srour et al, 1992). Use of this model to assess human HSPC function is, however, limited due to the high maintenance costs and restricted approval by the relevant committees.

1.3 Haemopoietic stem cell transplantation (HSCT)

1.3.1 History of HSCT

The modern clinical treatment of haemopoietic stem cell transplantation to myeloablated patients has its roots in the 1950s when Lorenz and colleagues observed that the lethal effects of ionising radiation on mice can be avoided if the animals are given an intravenous injection of BM from a donor animal (Lorenz et al, 1951). It was later established that this protective effect was related to the transplanted cells (Barnes & Loutit, 1954). This was a particularly exciting discovery which meant that higher, potentially lethal, doses of cytotoxic drugs could be used to treat malignancies, followed by transplantation of donor cells to reconstitute haemopoiesis.

Translation of that theory in a clinical trial was promptly attempted with disappointing results initially, as successful long-term engraftment was possible for only one patient and everyone else in that initial trial died of progressive disease or allograft failure (Thomas et al, 1957). A later trial using donor grafts from identical twins to treat leukaemia underlined the need for cytotoxic treatments (in addition to irradiation) to prevent recurrence of leukaemia (Thomas et al, 1959). In the same year autologous transplantation was attempted by McGovern and co-workers, using cryopreserved cells from a previous remission of the patient (McGovern et al, 1959). More encouraging data were presented in reports that showed retention of allogeneic grafts (Beilby et al, 1960; Mathé et al, 1965) and from experiments in larger animals
(Cavins et al, 1964). Although the initial aim was the safe escalation of myeloablative treatments it was soon discovered, from retrospective analysis of the clinical data, that the grafts had an additional advantage; they induced a “graft-versus-tumour” effect.

Later discoveries of cytotoxic drugs cyclophosphamide and busulphan, as well as better methods for acquiring grafts, including the discovery of PB mobilisation improved the outcomes of the clinical trials (discussed in section 1.3.5.2). The histocompatibility problem, however, had yet to be addressed and that constituted the major hurdle that prevented the success of these treatments, due to immune graft rejection. This was first addressed in canine models (Epstein et al, 1968; Storb et al, 1971) and it underlined the need for immunomodulatory drugs (Lochte et al, 1962; Storb et al, 1970). Suppression of the host’s immune system was soon attempted in humans which allowed for successful engraftment of donor cells without graft rejection (Thomas et al, 1971; Santos et al, 1971).

1.3.2 Autologous, syngeneic and allogeneic transplantation

Due to histocompatibility issues, the use of healthy, HLA-matched donors is generally preferred for HSPC transplantation. This is referred to as allogeneic transplantation. When HSPC are provided by an identical twin sibling this is referred to as syngeneic graft. The degree of HLA matching between host and donor directly impacts on the success of allogeneic transplantation.

Siblings are more likely to be HLA-matched than non-related donors, although fully-matched siblings are only identified for 25-35% of patients. HLA-matched siblings are preferred over matched unrelated donors because of the risk of unknown, and hence unmatched, antigens in the latter case. Due to the constantly increasing BM registries the chances of locating an unrelated HLA-matched donor are improving. In general, the possibility of locating a matched donor with a different racial background is very rare, due
to racial segregation of HLA types. In all other cases where HSPC transplantation is required, autologous transplantation is usually performed. As there is no risk of Graft Versus Host Disease (GVHD) and weaker immunosuppression suffices, this treatment can be used for comparatively older patients. Moreover, autologous transplantation requires less toxic regiments and has faster lymphorecovery kinetics. Mononuclear cells from the patient are collected prior to myeloablation. This is sometimes followed by a selection of the CD34+ cells. After the cytotoxic treatment is completed the autologous graft is returned to the patient. Although this method eliminates the risk of GVHD, reoccurrence of the malignancy is more frequent as there is no graft-versus-tumour effect.

1.3.3 HSCT uses today

Today HSCT has become common practice with approximately 30,000-40,000 procedures performed worldwide, yearly. This number has increased by 10-20% every year since the 2000s. HSCT is used for the treatment of several haematological cancers as well as other tissue malignancies, metabolic disorders and immunodeficiencies (Hematopoietic Stem Cell Transplantation, 2012).

Haematological malignancies treated with autologous HSCT include plasma cell disorders, multiple myeloma, non-Hodgkin’s lymphoma, Hodgkin lymphoma and less commonly acute myeloid leukaemia (AML). Allogeneic transplantation is the preferred treatment when HSCT is used for acute lymphoblastic leukaemia, AML, chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia, myeloproliferative disorders and myelodysplastic syndromes and less frequently Non-Hodgkin’s Lymphoma. The choice of treatment, however, depends on a number of parameters including the age of the patient, previous relapses, responsiveness to chemotherapy and the availability of a matched donor.
In AML, for example, HSCT is the first treatment of choice in paediatric patients whereas in adults use of this treatment depends on the risk stratification of the patient (Niewerth et al., 2010). Primary refractory and relapsed AML are generally only cured by allogeneic HSCT.

In CML, because of the discovery of imatinib (a receptor tyrosine kinase inhibitor), HSCT is generally only recommended to young (<20 years) patients with grafts from matched donors and for patients younger than 40 years old only if an HLA-identical sibling donor is available.

Myelodysplastic syndromes are treated with allogeneic HSCT from HLA-matched sibling donors for most patients below the age of 60 years.

In Hodgkin’s lymphoma, autologous transplantation (combined with high-dose chemotherapy) is preferred for high risk patients or after early relapse following initial chemotherapy treatment, depending on disease status. Similarly, in non-Hodgkin’s lymphoma, autologous or allogeneic HSCT, with chemotherapy, is advised after relapse or if the primary therapy does not achieve complete remission.

Multiple myeloma is currently not a curable disease but autologous HSCT can prolong event-free and overall survival by a year compared with chemotherapy alone. Allogeneic transplantation in this disease is associated with higher mortality rate whereas double autologous HSCT appears to improve survival (over single autologous HSCT).

Additional malignancies in which HSCT has been attempted, with varying results, include breast cancer, neuroblastoma, testicular cancer, sarcoma and germ cell tumours. Other conditions that are treated with autologous HSCT include autoimmune disorders and amyloidosis. Allogeneic HSCT is used for the treatment of several anaemias, thalassaemia major, Wiskott-Aldrich syndrome and metabolic disorders, such as mucopolysaccharidosis. HSCT has also been increasingly used to treat immunodeficiency syndromes (such
as severe immunodeficiency syndrome-SCID). Ongoing clinical trials for immunodeficiencies utilise HSCT combined with gene correction of the graft prior to transplantation. These protocols normally induce proliferation of the HSPC (with cytokines) and then use viral transduction to deliver the required gene. The autologous gene-corrected graft is then delivered to the patient.

1.3.4 Factors influencing HSCT success

The use of HSCT and its success rates vary considerably depending on type of disease as well as its status (relapse/remission). It is also related to any cytogenetic abnormalities the patients may carry as well as their response to previous therapy. Other factors include the age of the patient and specific evidence related prognostic factors, including genetic lesions.

Allogeneic HSCT success is also directly related to the HLA matching between the donor and the recipient. Matching of all 6 basic HLA antigens is more likely to result in successful transplantation whereas less strictly matched grafts may lead to graft rejection of GVHD.

Moreover, the intensity of the conditioning delivered to the patients prior to transplantation is also related to the success of this treatment. High intensity conditioning is mainly used before transplantation of unrelated, matched donor grafts to ensure immunosuppression and engraftment. On the other hand, this regime increases the incidence of graft-versus-host disease as well as infectious complications post transplantation.

Another important contributing factor to HSCT success is the cell dose delivered to the patient. This is important because small doses lead to delayed haemopoietic reconstitution and prolonged periods of neutropenia, which are associated with increased mortality. It is generally accepted that patients benefit from higher doses of HSPC (Siena et al, 2000), although the effect may be negligible above 8x10⁶ cells per kilogram (Sezer et al, 2000).
When smaller numbers of cells are available, infusion of more than $3.5 \times 10^6$ CD34$^+$ cells/kg minimises the risk of delayed platelet recovery. Doses between 1 and $3.5 \times 10^6$ CD34$^+$ cells/kg are associated with a 5-15% risk of delayed platelet recovery. If less than $1 \times 10^6$ CD34$^+$ cells/kg are transplanted there is approximately 40% risk of delayed immune recovery, often leading to prolonged thrombocytopenia (Watts & Linch, 1997).

1.3.5 Sources of HSC

1.3.5.1 Bone Marrow

Historically, BM was the first source that was used for HSPC transplantation. Procedure-related deaths in donors are rare (0.3%) (Anderlini et al., 2001) and local discomfort is minimal after a maximum of two weeks. Collection of these cells from the donor, however, is an invasive process that requires repeated aspiration of the posterior iliac crests, under general anaesthesia.

1.3.5.2 Peripheral Blood

It was known since 1975 that HSPC circulate, albeit in small numbers, in PB (Barr et al., 1975). The number of circulating progenitors, however, can be greatly increased after administration of chemotherapy and/or cytokines. This process of inducing HSPC release from the BM is termed PB mobilisation and G-CSF is the most widely used cytokine for this purpose (Watts et al., 2000), as discussed in section 1.7. More recently, combination of G-CSF with AMD3100 (a small molecule inhibitor of CXCR4, marketed by Genzyme under the trade name Plerixafor) was shown to significantly increase the numbers of HSPC in the circulation (Flomenberg et al., 2005). These mobilisation treatments allow for the collection of HSPC for transplantation from PB, in a minimally invasive procedure. HSPC are collected in leukapheresis procedure, whereby up to 25 litres of blood can be processed in 4 hours. This protocol, which was first attempted in the 1970s (Goldman et
al, 1978), is now the preferred method of obtaining donor (and patient) HSPC for transplantation. Clinical selection of CD34+ cells from PB is often performed prior to HSCT.

1.3.5.3 Umbilical Cord Blood (CB)

Blood collected from the umbilical cord and the placenta immediately after birth is rich in HSPC. This source was first used in 1989 to treat a case of Fanconi’s anaemia (Gluckman et al, 1989) but has been increasingly used so that today, thousands of patients have been transplanted with CB HSPC. Banks of CB cells have been set up all over the world (storing more than 200,000 units) and thus availability of these cells has improved. Moreover, the HLA-matching required is less stringent as CB grafts are less likely to cause GVHD, while appearing to maintain the graft-versus-tumour effect (Wagner et al, 2002). On the other hand the volume of blood collected from the umbilical cord, and subsequently the number of cells, are low (rarely more than a few million cells per graft). As a result individual grafts are only appropriate for paediatric patients whereas adult patients are usually transplanted with two or more grafts (Barker et al, 2005). The reduced cell numbers lead to slow haemopoietic (and immune) reconstitution, which increases the risk of infection post-transplantation.

1.3.5.4 Choice of cell source for HSCT

The functional qualities of BM, MPB and CB HSPC are not identical. Two groups have reported that, on a “per cell” basis, CB cells engraft 10-15 times more efficiently in xenogeneic models compared to BM HSPC (Van der Loo et al, 1998a; Holyoake et al, 1999). BM HSPC, in turn, appear to proliferate and differentiate more readily in comparison to their MPB counterparts (Prosper et al, 1996) and their “per cell” reconstitution capacity is superior to that of MPB HSPC, in xenogeneic models (Verfaillie et al, 2000).
In comparison to BM derived grafts MPB contain more T cells and are thus more likely to induce GVHD (Cutler et al, 2001), increasing mortality and morbidity in allogeneic transplantation (Stewart et al, 2004). Moreover, in autologous transplantation, the G-CSF treatment sometimes fails to mobilise an adequate number of HSPC (10-20% of patients), especially if the patient has previously received intensive chemotherapy (when this percentage increases to 40%) (Kessinger & Sharp, 2003). On the other hand, this treatment does not require anaesthesia, is less invasive and produces more rapid haemopoietic reconstitution in the recipients (Talmadge et al, 1997).

The choice of cell source used for HSCT is mainly dependent on availability and the particular disease characteristics. When, for example, there is high risk of GVHD BM cells are preferred, whereas when a “graft-versus-tumour” effect is desirable MPB HSPC are used. Overall, however, MPB HSPC are the most widely used cell source for HSCT due to their abundance, availability and the fast reconstitution kinetics, which are related to their comparatively higher concentration of “short-term repopulating cells” (Körbling & Anderlini, 2001). In AML, which is a good indicator for the use of allogeneic HSCT, 93% of the HLA-identical sibling grafts (Nagler et al, 2012a) and 84% of the matched unrelated grafts (Nagler et al, 2012b) were harvested from MPB.

1.4 Stem cell homing and engraftment

After infusion of the HSPC in the recipient, the cells circulate and home to the haemopoietic niches. They then need to tether onto, roll and transmigrate through the BM sinus endothelium. The process by which HSPC enter the BM from the moment of infusion is called homing. Through a variety of adhesive interactions (discussed in section 1.6) the cells then attach onto the microenvironment of the niches. This process is followed by the long-term retention and proliferation/self-renewal of the HSC in these sites which is
termed engraftment. This is a multi-step process (Figure 1.2) that is affected by a variety of factors, described below. Clinically, engraftment in the recipient is defined as a neutrophil count of >0.5x10^9/litre and a platelet count of >20 x10^9/litre for 3 successive days.

1.4.1 Tethering and rolling

Circulating HSPC form a loose and transient adhesive interaction with the endothelium (tethering). This process is mediated by selectins and their ligands. More specifically it has been shown to be dependent on P-selectin (expressed on platelets and activated endothelial cells) and E-selectin (expressed on endothelial cells) (Mazo et al., 1998; Frenette et al., 1998; Papayannopoulou et al., 2001). L-selectin (expressed on leukocytes) has also been implicated but appears to have a lesser role in progenitor tethering (Mazo et al., 1998). Due to the shear force applied on the tethered progenitors by the flowing blood, tethering is followed by rolling on the endothelial surface. HSPC express a variety of integrins on their surface, which are functionally inactive. Rolling allows them to “sample” the local endothelium for chemokines that will activate these integrins (Figure 1.2 A and B). Binding of endothelial chemokines to seven transmembrane-spanning G-protein coupled receptors on the HSPC (Rollins, 1997) induces conformational changes in the integrin heterodimer (Ginsberg et al., 1992) which results in higher affinity of the integrins to their ligands on the endothelial surface (Lévesque et al., 1995). Such activation will eventually initiate the transmigration cascade (Figure 1.2 C). These interactions involve integrins such as VLA-4 and LFA-1 (and their ligands VCAM-1 and ICAM-1 respectively) and they are stimulated by SDF-1 which is expressed on endothelial cell surface or bound in the endothelial extracellular matrix (Peled et al., 2000). If activating molecules such as SDF-1 are absent the adhesive interactions break and the HSPC are released back into the circulation (Figure 1.2 Bi).
Figure 1.2: HSPC rolling, firm adhesion and transendothelial migration. (A) HSPC interact with the constitutively expressed E and P selectins on endothelial cells and the rolling process is initiated. Rolling, CXCR4+ HSPC 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 that bind to fibronectin. (D) Migrating HSPC will eventually reach the haemopoietic tissue where they will adhere to stromal cells, ECM proteins, nestin+ MSC or osteoblasts. (Modified from Peled et al., 2000 and the thesis of F. Ahmed, 2004).
1.4.2 Transmigration

When strong adhesion between the HSPC and the endothelial layer occurs, the former move towards the “cell-cell junctions” of the endothelium (Möhle et al, 1999). The HSPC then undergo a number of morphological and receptor expression changes which include polarisation of actin and signalling groups that lead to polarised adhesion (Figure 1.2 C). They also form lamellipodia and through actomyosin contractility travel towards the chemokine gradient reaching the subendothelial basal membrane in the process (diapedesis) (Imhof & Dunon, 1995). This movement is dependent on the interaction of PECAM-1 ligand (which is accumulated at the endothelial cell junctions) with the αvβ3 integrin on HSPC (Yong et al, 1998). SDF-1, which is produced by several types of BM cells (Maekawa & Ishii, 2000) and exists in abundance in the bone cavities, is essential for the migration of HSPC (Peled et al, 1999a; Imai et al, 1999; Wright et al, 2002). It exerts its effect on HSPC mainly through the CXCR4 receptor (discussed in sections 1.6.6 and 1.7.3). Blockade of the CXCR4 receptor has been repeatedly shown to inhibit HSPC migration in vitro as well as homing and engraftment in vivo thus underlying the importance of this chemokine/receptor pair in these processes (Oberlin et al, 1996; Peled et al, 1999b; Kollet et al, 2001). Transmigration, in this context, is completed when the cells have entered the BM sinus. That also completes the homing process.

1.4.3 Adhesion and long-term retention in the niches

Upon entering the BM space the HSPC localise in proximity to endosteal or vascular tissue depending on their differentiation status. In irradiated animals primitive HSPC were shown to anchor in proximity to endosteal surfaces (Lo Celso et al, 2009; Xie et al, 2009) whereas more differentiated cells are
reported to localise closer to the vascular tissue at the centre of the marrow (Heissig et al, 2002; Avecilla et al, 2004; Kopp et al, 2005). Retention in these sites occurs through a number of adhesion receptors (Figure 1.2 D). The most well-established receptor/ligand pairs include VLA-4 and VLA-5 with VCAM-1 and fibronectin, CD44 with osteopontin, fibronectin, collagen, laminin and hyaluronic acid, LFA-1 with ICAM-1, CXCR4 with SDF-1 and N-cadherin (homotypically) (discussed in detail in section 1.6).

Anchorage of transplanted HSPC within the niches is essential for their long-term retention at these sites. The retention, proliferation and/or self-renewal of the attached cells in haemopoietic tissue constitute stem cell engraftment.

1.5 Haemopoietic niches

1.5.1 Sites

The viability, differentiation and function of HSC directly depend on the specific conditions of the microenvironment surrounding and supporting them. In developing mammalian embryos haemopoiesis occurs sequentially in the yolk sac, foetal liver, spleen, lymph nodes and finally the BM. In addition to these sites large populations of granulocytes are found in connective tissue for most of foetal development. Large numbers of HSC are also found in foetal circulation as well as in umbilical CB. After birth, haemopoietic activity is restricted to the BM (except for some specific disease states). This happens at different times for different bones and is related to ossification. In children, blood formation takes place mainly in the long bones whereas in adults, areas such as the pelvis, cranium, sternum and vertebrae are more active.
1.5.2 Supporting cells

The BM microenvironment comprises a variety of cells which support survival and growth of HSC. Early research demonstrated that these cells mediate the self-renewal versus differentiation decisions of the HSC (Trentin, 1971; Schofield, 1978). Recent studies have identified specialised cell populations and their impact on the maintenance, expansion and/or differentiation of HSC (Zhang et al, 2003; Calvi et al, 2003; Avecilla et al, 2004; Blank et al, 2008; Méndez-Ferrer et al, 2010). This specific, multi-functional environment is defined as the HSC niche. When outside the niches HSC cannot self-renew and they initiate differentiation processes according to the surrounding molecular signals, to produce mature blood cells. The cell types found in the BM niches include stromal cells such as fibroblasts, endothelial cells, macrophages, adipocytes, as well as osteoblasts and osteoclasts. More primitive cells such as the nestin+ mesenchymal stem cells (MSC) have also been shown to support and control the proliferation of HSC.

1.5.3 Non-cellular components

The stromal cells produce a variety of proteins that form the extracellular matrix (ECM) of the BM which aids migration and adhesion of the HSC. These proteins include collagen, laminin, glycoproteins (fibronectin and thrombospondin) and glycosaminoglycans (hyaluronic acid and chondroitin derivatives).

In addition to the ECM, a variety of growth factors are secreted by stromal cells and are present in the BM niches where they regulate haemopoiesis (Table 1.3). Stem cell factor and Fms-like tyrosine kinase 3 Ligand (Flt3L) act on pluripotent HSC, whereas Interleukin-3 and -6 (IL-3, IL-6) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) act on more mature, multipotent HSC. Granulocyte and Macrophage Colony-Stimulating Factors (G-CSF and M-CSF), Interleukin-5 (IL-5), Erythropoietin (EPO) and
<table>
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<tr>
<th>Cytokine</th>
<th>Differentiation stage</th>
<th>Function</th>
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<tr>
<td>IL-1</td>
<td>Early &amp; late</td>
<td>CMP &amp; T-lymphocytes</td>
</tr>
<tr>
<td>IL-2</td>
<td>Late</td>
<td>Pre-T-cells, T-lymphocytes</td>
</tr>
<tr>
<td>IL-3</td>
<td>Early &amp; late</td>
<td>CMP &amp; megakaryocytes, erythrocytes, basophils, neutrophils, eosinophils, monocytes</td>
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<tr>
<td>IL-4</td>
<td>Late</td>
<td>T-lymphocytes</td>
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<td>IL-5</td>
<td>Late</td>
<td>Eosinophils</td>
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<tr>
<td>IL-6</td>
<td>Early &amp; late</td>
<td>CMP &amp; T-lymphocytes, basophils, neutrophils, monocytes</td>
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<tr>
<td>IL-7</td>
<td>Late</td>
<td>T-lymphocytes</td>
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<td>IL-11</td>
<td>Late</td>
<td>Platelets, megakaryocytes</td>
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<td>IL-12</td>
<td>Early</td>
<td>Pre-T-cells</td>
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<td>SCF</td>
<td>Early &amp; late</td>
<td>CMP &amp; megakaryocytes, erythrocytes, basophils, neutrophils, monocytes</td>
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<td>EPO</td>
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<td>Flt3L</td>
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<td>TPO</td>
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<td>G-CSF</td>
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<td>GM-CSF</td>
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<td>CMP &amp; megakaryocytes, erythrocytes, myeloblasts, basophils, neutrophils, eosinophils, monocytes</td>
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<td>M-CSF</td>
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<td>SDF-1</td>
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<td>TNF-α</td>
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<td>TGF-β</td>
<td>Early</td>
<td>Pre-T-cells</td>
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**Table 1.3: Haemopoietic cytokines.** The stages in HSC differentiation on which they act as well as the cell type produced after relevant stimulation are shown. Interleukins (IL), Stem Cell Factor (SCF), erythropoietin (EPO), Fms-like tyrosine kinase receptor Ligand (Flt3L), thrombopoietin (TPO), Granulocyte/Monocyte-Colony Stimulating Factor (G/M/GM-CSF), Stromal Cell-Derived Factor-1 (SDF-1), Tumour Necrosis Factor-α (TNF-α) and Transforming Growth Factor-β (TGF-β).
Thrombopoietin (TPO) act on oligopotent and differentiated cells. All these cytokines are produced in the BM (with the exception of EPO which is mainly synthesised in the kidney) and control the fate of HSC in the niches. Relevant haemopoietic growth factors are dealt with, in more detail in Chapter 3.

1.5.4 Oxygen gradient

HSC preferentially use anaerobic metabolic pathways; oxidative phosphorylation is reduced while glycolytic activity (for ATP synthesis) is increased. Thus, little energy is required for HSC to be maintained in hypoxic conditions. Moreover, slow cycling and quiescent HSC are mainly located in hypoxic areas in the BM and away from the capillaries. On the contrary cycling and differentiated haemopoietic progenitors reside closer to normoxic areas. Altogether, hypoxia has been associated with HSC maintenance and self-renewal and is, therefore, an essential component of the HSC niches. HSC respond to hypoxia through upregulation of the hypoxia-inducible factor (HIF)-1. HIF-1, in turn upregulates a variety of other genes including cytokines and transcription factors that maintain HSC multipotency.

1.5.5 Osteoblastic and Vascular niches

The bone in the BM is a multi-cellular tissue which includes mesenchymal stem cells, osteoprogenitor cells, osteoblasts and osteocytes, in differentiation order (Mackie, 2003). The proportions of these cells and the general architecture of the bone is tightly controlled by the balance between bone formation, by osteoblasts and bone resorption, by osteoclasts (Martin & Sims, 2005). The osteoblasts, which are located in the endosteum in vivo, were shown in early in vitro studies to sustain HSC (Taichman & Emerson, 1994; Taichman et al, 1996; Taichman & Emerson, 1998; Taichman et al, 2000). More recent in vivo reports established these cells as major components of the haemopoietic niche (Calvi et al, 2003; Zhang et al, 2003). While osteoblast expansion has been demonstrated to correlate with HSC
numbers, contradicting data exist for the consequences of osteoblast reduction in vivo. Visnjic and colleagues reported direct reduction in haemopoiesis after osteoblast depletion (Visnjic et al, 2004) whereas Kiel and co-workers report no such effect (Kiel et al, 2007). Osteoblasts exert their effect on HSC proliferation through the production of a variety of positive (Angiopoietin-1, TPO and Jagged-1) and negative (osteopontin and Dikkopf1) regulators. Importantly, osteoblasts also produce SDF-1 which has well described roles in HSC homing, migration, adhesion and survival (Broxmeyer et al, 2005).

Vascular endothelial cells line all the vessels in the body (from the heart to the small capillaries). In the BM they form a layer separating the HSC from the circulation. Haemopoietic cells entering or leaving the BM cavities have to come in contact with the vascular endothelial cells (Winkler & Lévesque, 2006). Two simultaneous studies suggested that HSC may also reside and proliferate in perivascular sites (Kiel et al, 2005; Sipkins et al, 2005). This is supported by in vitro studies demonstrating that endothelial cell lines support HSC survival (Rafii et al, 1995; Chute et al, 2002; Li et al, 2004). Recent in vivo data shows that inhibition of the endothelial production of SCF is enough to significantly reduce HSC numbers in the BM (Ding et al, 2012).

A number of groups have reported that endosteal niches hold the more primitive, quiescent HSC (Zhang et al, 2003; Arai et al, 2004, 200; Wilson et al, 2004, 2007; Yoshihara et al, 2007; Haug et al, 2008). The vascular niches, on the other hand have been proposed to sustain the more differentiated or activated HSC (Kopp et al, 2005) (Figure 1.3). This was first suggested by Rafii and co-workers (Rafii et al, 1995) but was shown more specifically by Avcèilla et al in 2004. In this report megakaryocyte progenitors were shown to mature and differentiate when in contact with vascular sinusoids (Avcèilla et al, 2004). Overall the literature suggests that the osteoblastic niches control the viability and proliferation of HSC while the vascular microenvironment has
a stimulatory role (Perry & Li, 2007). In the diaphysis of the BM the vascular environment is devoid of osteoblasts (De Saint-Georges & Miller, 1992). Despite this observation, it is highly likely that, to a great extent, these two components are not actually distinct or distant in vivo and that they form a common microenvironment.

1.6 HSPC adhesion receptors and ligands

HSPC have been shown to intimately interact with the BM cavities and to form cellular aggregates (Funk et al, 1994). In long-term BM cultures, in vitro, the most primitive HSPC are found within the adherent layer (Coulombel et al, 1983). These data suggest that the adhesive interactions between HSPC and their microenvironment are essential for their maintenance and self-renewal. In vivo, however, in-depth analysis of HSPC attachment was initially restricted by the technologies available to detect donor cells in the recipient. Early attempts included immunophenotyping of the donor cells, congenic models, fluorescent in situ hybridisation (FISH) and detection of the Y chromosome of male donor cells in female recipients (Lamar & Palmer, 1984; Slezak & Muirhead, 1991; Spangrude et al, 1995; Nilsson et al, 1996, 1998; Insern & Méndez-Ferrer, 2011). These protocols were limited by low sensitivity and specificity or high background signal. Fluorescent dyes were most informative regarding the lodgment of transplanted cells in the BM (Lanzkron et al, 1999), although this technique is restricted to short-term analysis because cell fluorescence is halved with every cell division. Further reports using fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Nilsson et al, 2001) or real-time live imaging (Lo Celso et al, 2009; Xie et al, 2009) demonstrated that primitive cells tend to localise closer to the endosteum and to osteoblasts whereas more mature progenitors were retained increasingly further away, to the vascular zone at the centre of the marrow from where they re-establish haemopoiesis (Heissig et al, 2002; Avecilla et al, 2004; Kopp et al, 2005) (Figure 1.3).
Figure 1.3: Homing and mobilisation to and from the endosteal and vascular niches. Homing HSPC transmigrate through the endothelial lining of the sinuses and lodge at the vascular or endosteal niches, where they interact with site-specific adhesion ligands. Resident HSPC are mobilised or physiologically recruited to the circulation from the vascular niches, which are located closer to the sinusoids. Quiescent HSC are considered to reside at the endosteal niches, where the oxygen concentration is lower, whereas primed HSC are found at the vascular niches and are exposed to higher oxygen concentrations.
Although the signals exchanged between HSPC and the supportive environment are not fully described, a number of molecules have been suggested to play a role in their retention in endosteal or vascular niches.

1.6.1 N-cadherin

N-cadherin is a type-1 transmembrane protein typically found in neurons, that has been shown to play an important role in Ca\(^{2+}\)-dependent cell adhesion, mainly through homotypic interactions (reviewed in Derycke & Bracke, 2004). In 2003 and 2004, when the role of osteoblastic cells as niche components was being established, two groups published reports showing that a population of spindle-shaped osteoblasts are in contact, through homotypic interactions, with HSC which also express N-cadherin (Arai et al, 2004; Zhang et al, 2003). The functional role of N-cadherin was not described in these studies and in fact further studies showed that N-cadherin may not be essential for HSC maintenance (Kiel et al, 2007; Kiel & Morrison, 2008). At the same time Hosokawa et al demonstrated that suppression of the N-cadherin based cell adhesion (by ROS) causes HSC egress from the niches. Haug and colleagues successfully detected low N-cadherin levels in primary HSC (Haug et al, 2008). The next year Kiel and co-workers published another paper reporting no N-cadherin expression in two populations of primary murine HSC (Kiel et al, 2009). These contradicting data were further complicated by the use of the monoclonal antibody MNCD2 for N-cadherin detection which was later shown not to be specific (Haug et al, 2008; Kiel et al, 2009; Li & Zon, 2010). The increasing controversy lead to the production of a review that attempted to organise an experimental debate between the disagreeing groups (Li & Zon, 2010). As the set experimental design was not sufficiently followed by the two main groups the report concludes that N-cadherin may be expressed by a sub-set of HSC at very low levels and that further in vivo work is necessary to establish its role in the niche. This confirmation came with a later report by the Suda group, which demonstrated
that N-cadherin knockdown in murine HSC reduced long-term engraftment of these cells to the osteoblastic niche (Hosokawa et al, 2010).

### 1.6.2 CD44 receptor

CD44 is a class-I transmembrane glycoprotein that mediates cell-cell and cell-ECM interactions. It was first identified as an antigen expressed on human white blood cells and was later shown to belong to a group of proteins that are expressed on most vertebrate cells (reviewed in Ponta et al, 2003). It has been shown to have roles in matrix adhesion, vascular extravasation, T-cell co-stimulation, and lymphocyte activation. CD44 protein exists in many heterogeneous isoforms, all of which are encoded by a single gene. The heterogeneity of the proteins is related to post-translational modifications and to alternative splicing. The pre-mRNA of CD44 has 20 exons, 10 of which are regulated by alternative splicing. The smallest and most widely expressed isoform contains exons 1–5 and 16–20 and is termed CD44s. Larger isoforms are referred to as CD44v and a few epithelial cells also express CD44E, an isoform that includes exons 8-10. CD44 is expressed on haemopoietic cells of mesodermal origin during embryogenesis and in the adult organism (Stamenkovic et al, 1991). In the context of haemopoietic niches, CD44 mediates the adhesion between HSPC, BM progenitors and stromal cells (Miyake et al, 1990; Verfaillie et al, 1994b; Wagner et al, 2008); when blocked, lympho-haemopoiesis is inhibited (Miyake et al, 1990). CD44 has been shown to interact with a variety of ligands within the BM microenvironment which are further discussed below.

#### 1.6.2.1 Osteopontin

One of the main ligands for CD44 within the BM is osteopontin (Weber et al, 1996; Katagiri et al, 1999). Osteopontin is a small integrin-binding, N-linked glycoprotein (SIBLING) which is expressed by osteoblasts, pre-osteoblasts, osteocytes as well as other haemopoietic cells in the BM (Mark et al, 1987;
Swanson et al, 1989). Binding of HSPC to osteopontin has been shown to be a potent constraining factor on their proliferation (Nilsson et al, 2005). More recently osteopontin was also shown to be a powerful chemoattractant for HSPC (Grassinger et al, 2009). Osteopontin also binds the VLA-4 receptor (Barry et al, 2000), which is described in section 1.6.3.

1.6.2.2 Fibronectin

Another well-established ligand for CD44 within the haemopoietic tissue is fibronectin (Jalkanen & Jalkanen, 1992). Fibronectin is a glycoprotein which is a major component of the extracellular matrix (Verfaillie et al, 1994a; Long, 1992; Adams & Watt, 1993) and has specific functional roles. In the BM cavities fibronectin has been shown to localise in the endosteum, the central marrow region, the bone and the periosteum (Nilsson et al, 1998). Binding to fibronectin has been shown to aid maintenance, self-renewal and expansion of HSPC (Dao et al, 1998; Yokota et al, 1998; Schofield et al, 1998). Fibronectin can also interact with VLA-4 (Williams et al, 1991) and VLA-5 (Van der Loo et al, 1998), which are discussed in sections 1.6.3 and 1.6.4.

1.6.2.3 Hyaluronic acid

Hyaluronic acid is a single-chain high-molecular-mass glycosaminoglycan which is synthesised by hyaluronic acid synthases (Watanabe & Yamaguchi, 1996; Shyjan et al, 1996; Spicer et al, 1997). It is present in a variety of tissues and is a part of the non-protein glycosaminoglycan component of the BM extracellular matrix (Dorshkind, 1990; Siczkowski et al, 1993). It is described to be particularly highly expressed around trabecular bone, within the metaphysis (Ellis et al, 2011). Its role in haemopoiesis was first described by the Nilsson group who showed that it is essential for HSPC attachment to the endosteum post-transplantation, while it is also involved in the proliferation and differentiation of these cells (Nilsson et al, 2003). Recently, the same group reported that blockade of CD44 on hCD34+ cells or pre-
treatment with soluble hyaluronic acid, significantly decreased homing of the transplanted cells to immunodeficient mice (Ellis et al., 2011). This observation is in agreement with previous reports using hyaluronidase (Avigdor et al., 2004).

1.6.2.4 Collagen and laminin

The interaction of the CD44 receptor with collagen was first described in melanoma cells (Faassen et al., 1992; Knutson et al., 1996). CD44 binding to laminin was confirmed with ELISAs by Jalkanen et al., who also showed binding to collagen Type I and fibronectin (Jalkanen & Jalkanen, 1992). Both collagen and laminin are present in the BM vessels, arterioles, veins, and sinuses and on bone (Nilsson et al., 1998). Additionally, certain types of collagen are also found on the endosteum and periosteum (Nilsson et al., 1998). Other receptors for collagen include VLA-1, VAL-2 and VLA-3 (Elices et al., 1991; Richter et al., 2007).

1.6.3 Very Late Antigen (VLA)-4

VLA-4 is a heterodimeric integrin composed of an α (CD49d) and a β (CD29) subunit. VLA-4 expression has been detected on mononuclear leukocytes as well as on some non-haemopoietic cells (Hemler et al., 1990). Murine (Orschell-Traycoff et al., 2000) and human CD34+ cells (CB, BM, and MPB) have been shown to express VLA-4 (Saeland et al., 1992; Liesveld et al., 1993; Leavesley et al., 1994; Denning-Kendall et al., 2003). In murine cells, blockade of VLA-4 with a monoclonal antibody completely inhibited lymphopoiesis and delayed myelopoiesis in long-term BM cultures (Miyake et al., 1991). Moreover, development of erythroid colonies on stromal cells is also inhibited by VLA-4 blocking antibodies (Yanai et al., 1994). Similarly, human progenitor colony growth on supportive stromal cell lines was reduced by a specific anti-VLA-4 antibody (Ryan et al., 1992). In vivo, homing of BM HSPC is significantly reduced after VLA-4 blockade in murine
(Papayannopoulou et al., 1995) and sheep models (Zanjani et al., 1999). VLA-4 interacts with a variety of ligands within the BM such as fibronectin (Williams et al., 1991), osteopontin (Barry et al., 2000), which are discussed above and VCAM-1 discussed in section 1.6.3.1. It also mediates CD44 binding to hyaluronic acid (Siegelman et al., 2000).

1.6.3.1 VCAM-1

One of the main BM ligands that mediates HSPC attachment in the niches via its interaction with VLA-4 is the Vascular Cell Adhesion Molecule-1 (VCAM-1) (Simmons et al., 1992). VCAM-1 is a cell surface sialoglycoprotein which is expressed by human and murine BM endothelial cells (Schweitzer et al., 1996; Jacobsen et al., 1996). VLA-4 binds strongly to VCAM-1 (Lobb & Hemler, 1994) and this interaction is one of the major adhesive forces between HSPC and the BM stroma (Simmons et al., 1992; Oostendorp et al., 1995). Moreover, in in vivo models, blockade of VCAM-1 (or VLA-4) has been shown to significantly decrease lymphocyte homing to the BM, whereas the numbers of circulating HSPC are increased. In fact administration of anti-VLA-4 or anti-VCAM-1 antibodies induced HSPC release to the circulation in healthy animals (Papayannopoulou et al., 1995). In contrast to other receptors such as CD18, CD11a and L-selectin, whose blockade decreases but does not inhibit HSPC homing (Mazo et al., 1998; Vermeulen et al., 1998; Papayannopoulou et al., 2001) the VLA-4 interaction with VCAM-1 was shown to be essential for HSPC homing to the BM (Papayannopoulou et al., 2001).

1.6.4 Very Late Antigen (VLA)-5

VLA-5 is a dimeric integrin that binds to matrix macromolecules. It was first described in 1985 as the main receptor for fibronectin in an osteosarcoma cell line (Pytela et al., 1985). It has a wide range of expression (including platelets, lymphocytes, and myeloid, epithelial, endothelial cells and fibroblasts) and it is essential for the adhesion of these cells with extracellular matrix
components (Wayner et al, 1988). It is also expressed by long-term repopulating haemopoietic cells and it mediates their adhesion to fibronectin (Van der Loo et al, 1998b; Denning-Kendall et al, 2003). VLA-5 is also expressed in murine Sca-1^lin^- cells and it is essential for their long-term engraftment (Orschell-Traycoff et al, 2000). In addition to fibronectin, VLA-5 interacts with VCAM-1 on stromal cells (Teixidó et al, 1992). Blockade of VLA-5 significantly impairs the homing of human progenitors to the spleen and BM of immunodeficient mice (Kollet et al, 2001), but does not have a significant impact on BM repopulation, which implies a co-operative in vivo effect between this integrin and other pathways such as the proteoglycan/CD44 (Van der Loo et al, 1998b).

1.6.5 Lymphocyte Function-Associated antigen-1 (LFA-1) and Intracellular Adhesion Molecule (ICAM)

Another well described adhesion pathway is the LFA-1/ICAM pair, particularly for leukocytes (Wawryk et al, 1989). LFA-1 is an integrin with a β2 and an αL domain. It is expressed on T-cell populations as well as on B-cells, macrophages and neutrophils. It controls leukocyte adhesion (required for cytolytic conjugate formation), helper T-cell interactions and antibody-dependent killing (Simmons et al, 1988). The ligands for LFA-1 are three ICAM isoforms (Binnerts et al, 1994). LFA-1 expression was shown on primary myeloid progenitors (Kansas et al, 1990) and CB HSC (Denning-Kendall et al, 2003) while both LFA-1 and ICAM expression was demonstrated for BM and MPB hCD34^+ cells (Turner et al, 1995; Möhle et al, 1995). Although not as essential as VLA-4 and VLA-5, specific blockade experiments demonstrated that LFA-1 also participates in the engraftment of HSPC in the BM (Peled et al, 2000). The same group demonstrated that this is at least partly related to its role in HSPC homing to the BM. Blockade of LFA-1 reduced BM homing by 58 ± 16% (Kollet et al, 2001). Similar results were reported on the role of LFA-1 in HSPC homing to the spleen (Kollet et al,
2001) although Vermeulen had previously shown that LFA-1 blockade does not affect CFC homing to the spleen (Vermeulen et al, 1998). This discrepancy could be related to the different cell sources used (Kollet et al used human CB whereas Vermeulen et al used murine BM), different timing of the analysis (16 hours vs. 3 hours, respectively) or the different readout protocols (colony assays vs. serial transplantation).

Finally SDF-1 activation of LFA-1 has been shown to increase MPB lymphocyte adhesion to ICAM-1 (Campbell et al, 1998) and the same was observed by the Lapidot group for human CB CD34+ cells (Peled et al, 2000).

1.6.6 CXCR4 and SDF-1

CXCR4 is a seven-transmembrane spanning, G protein-coupled, alpha-chemokine receptor with specific affinity for SDF-1, although ubiquitin was recently reported as an additional CXCR4 ligand (Saini et al, 2010). It was originally cloned by three independent groups before it was associated to SDF-1 (Nomura et al, 1993; Federsspiel et al, 1993; Loetscher et al, 1994), but the focus on CXCR4 was initially centred around its role in HIV infection of permissive cells (Deng et al, 1997). It soon expanded, however, to the areas of lymphocyte trafficking and haemopoiesis. It is expressed on most leukocyte populations including the majority of T-lymphocyte subsets, all B cells and monocytes, and weakly on NK cells (Hori et al, 1998). CXCR4 expression has also been reported on primitive haemopoietic progenitors in murine models (Möhle et al, 1998) and on human HSPC of all sources (Aiuti et al, 1999a, 1999b; Kim & Broxmeyer, 1999; Denning-Kendall et al, 2003).

SDF-1 was first identified as the ligand for CXCR4 in 1996 (Oberlin et al, 1996; Bleul et al, 1996). The importance of the CXCR4/SDF-1 interaction in haemopoiesis was originally underlined by knockout models where either SDF-1 (Nagasawa et al, 1996) or CXCR4 (Zou et al, 1998; Ma et al, 1998) expression was abolished. These animals demonstrated decreased
production of B-cell progenitors in the liver and a generally abnormal B lymphopoiesis and myelopoiesis in the BM. Although the CXCR4/SDF-1 interaction is the main pathway through which chemotaxis and retention is mediated, SDF-1 also binds CXCR7. This interaction, however, appears to have an accessory role, mainly aiding CXCR4 mediated migration (reviewed in Thelen & Thelen, 2008).

Within the haemopoietic tissue, SDF-1 expression has been detected on several types of BM stromal cells, such as osteoblasts and vascular endothelial cells (Ponomaryov et al, 2000; Ara et al, 2003). It is through the CXCR4/SDF-1 interaction that the retention of haemopoietic progenitors in the BM is achieved (Nagasawa et al, 1996; Ma et al, 1999) and is therefore essential for the long-term engraftment of cells in the BM (Peled et al, 1999b). This was later confirmed by in vivo imaging, where murine HSPC were shown to attach to SDF-1 expressing vascular microdomains in the perivascular space (Sipkins et al, 2005).

The positive impact of SDF-1 in HSPC lodgment in the niches is at least partly related to the stimulating effect that it has on the integrin-mediated progenitor attachment on vascular endothelium. CB CD34+ cells exhibit increased attachment to HUVEC in the presence of SDF-1 and this effect was shown to be completely dependent on G-protein interactions (Peled et al, 1999a). More specifically, SDF-1 was shown to enhance adhesion to endothelium by activating the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways. In fact, endothelial adhesion was shown to be dependent on these interactions and sensitive to G-protein blockade (Peled et al, 1999a). These data were supported by another study which indicated that SDF-1 pre-incubation increased human MPB lymphocyte adhesion to TNF-activated HUVEC and VCAM-transfected CHO cells in a VLA-4 dependent manner (Grabovsky et al, 2000). In vivo, SDF-1 induced integrin activation on HSPC
results in increased engraftment (Peled et al, 2000) and proliferation (Lataillade et al, 2000) in immunodeficient mice.

In addition to the integrin mediated adhesion, SDF-1 has been shown to increase adhesion of CB CD34+ cells to hyaluronic acid and this effect is restricted to the most primitive repopulating stem cells (Avigdor et al, 2004).

The significance of the SDF-1/CXCR4 pathway in engraftment was underlined in competitive repopulation experiments where SDF-1 stimulation was shown to improve stem cell engraftment and to enhance myeloid progenitor survival (Broxmeyer et al, 2003; Ara et al, 2003).

1.6.7 Indirect adhesion-modulating pathways

In addition to the receptor-ligand pairs described, other pathways can modulate the adhesion of cells within the haemopoietic niches, indirectly. One of the best described such interactions is that of the c-Kit receptor with Stem Cell Factor (SCF). SCF is expressed in a number of tissues including fibroblasts and endothelial cells, while it is also detectable in MPB (Ashman, 1999). This cytokine exists as a transmembrane as well as a soluble protein, both of which forms are produced from alternative splicing of the same transcript and with proteolytic cleavage (Anderson et al, 1991; Broudy, 1997). Both types of the cytokine are necessary for haemopoiesis (Brannan et al, 1991). Distinct roles of SCF in proliferation and survival have been described for haemopoietic cell lines (Caruana et al, 1993; Miyazawa et al, 1995) and primary cells (Fujita et al, 1989; Toksoz et al, 1992). Membrane bound SCF from stromal cells has been reported to be a more persistent activator of tyrosine kinase, whereas its soluble counterpart induces downregulation and degradation of surface c-Kit (Miyazawa et al, 1995).

C-Kit is expressed on most primitive haemopoietic cells (reviewed in Wilson et al, 2007). Blockade of c-Kit significantly reduces short-term homing of
haemopoietic progenitors to the BM and spleen (Broudy et al., 1996) suggesting that interaction of SCF with the c-Kit receptor mediates HSPC attachment.

Other haemopoietic growth factors that are involved in the modulation of HSPC adhesion are thrombopoietin (TPO) and erythropoietin (EPO). HSPC express both c-MPL (Carver-Moore et al., 1996; Gotoh et al., 1997) and EPOR (Stopka et al., 1998), which are the receptors for TPO and EPO retrospectively. Exposure of haemopoietic cell lines to these cytokines has been shown to induce fibronectin adhesion through the VLA-4 and VLA-5 receptors (Gotoh et al., 1997).

Tie2 is a tyrosine kinase receptor initially detected on endothelial cells (Dumont et al., 1992) whereas Tie2 expression has also been detected on HSC (Iwama et al., 1993). Indeed, in foetal liver, the most primitive HSC belong in the Tie2 positive HSPC population (Hsu et al., 2001).

Angiopoietin-1 is a secreted glycoprotein that induces HSC adhesion to matrix components such as fibronectin and collagen through its interaction with Tie2 (Sato et al., 1998; Takakura et al., 1998). Exposure of HSC to Angiopoietin-1 has been shown to enhance their adhesion to osteoblasts in the murine system, in vivo (Arai et al., 2004).

1.7 Circulation and Mobilisation

1.7.1 HSPC circulation

HSPC are present in PB in very low numbers (Barr et al., 1975). They have been shown to extravasate into the circulation in a periodical fashion that follows the circadian rhythms, under physiological conditions (Méndez-Ferrer et al., 2008). More specifically, in mice, circulating HSPC strictly follow circadian fluctuations, peaking five hours after the initiation of light and reaching minimum levels 5 hours after darkness. Maximum HSC circulation
co-occurs with minimum SDF-1 levels in the BM microenvironment, whereas the inverse occurs when BM SDF-1 levels peak (Lucas et al., 2008). Circulating HSPC are recruited at sites of injury and for organ repair in a manner that involves their detachment from the BM microenvironment and transendothelial migration to enter the circulation. This is a multifactorial process which involves chemokine signaling, adhesion molecules and matrix-degrading proteases. The peak of HSC egress occurs simultaneously with the maximum bone remodeling activity (Simmons & Nichols, 1966).

While it is not clear why this regular release of HSC in the circulation occurs, its evolutionary advantage could be related with a repeated “filtering” of the HSC that are allowed to re-enter haemopoietic tissue. In fact, in Drosophila models, it has been shown that any foreign cells that find themselves in niches can de-differentiate and multiply (Kai & Spradling, 2003, 2004), an event which in the case of haemopoietic niches could produce the relevant malignancies. This “HSC filtering” hypothesis is supported by research in molecules that signal protection from “programmed cell death” which are downregulated in differentiated HSC. Such molecules include CD31 (Knepper-Nicolai et al., 1998; Brown et al., 2002), CD200 (Hoek et al., 2000; Copland et al., 2007), plasminogen activator inhibitor 1 (PAI1) (Park et al., 2008) and CD47 (Oldenborg et al., 2000; Blazar et al., 2001; Khandelwal et al., 2007). CD47, in particular, has been closely associated with the clearance of apoptotic or abnormal red blood and BM cells by splenic red pulp macrophages (Oldenborg et al., 2000; Blazar et al., 2001). Based on these observations, it can be hypothesised that, after mobilisation, only the more quiescent, “healthy” HSPC are allowed to transmigrate back into haemopoietic tissue. Overall, the physiological periodic release and re-engraftment of HSPC from and to the haemopoietic niches is tightly regulated, multifactorial process with potentially significant evolutionary consequences.
1.7.2 HSPC mobilisation

As discussed in section 1.3.5.2 the number of HSPC in PB is greatly increased after treatment of the patient/donor with chemotherapy and/or cytokines (mobilisation). The most potent and widely used cytokine for HSPC mobilisation is G-CSF (Watts et al., 2000). The SDF-1/CXCR4 pathway plays an important role in HSPC release from the BM (discussed in section 1.7.3). G-CSF acts by downregulating SDF-1 concentration in the BM while expanding myeloid and granulocyte progenitors. G-CSF-induced downregulation of SDF-1 is achieved by a decrease in SDF-1 transcription combined with proteolysis (by neutrophil proteases) of the SDF-1 produced from the BM stroma (Semerad et al., 2005). Regular use of G-CSF, often in combination with alternative mobilisation strategies (such as CXCR4 inhibition), have rendered MPB the preferred source of HSC for transplantation purposes in clinic, due to its accessibility and abundance in cell numbers compared to BM and CB.

1.7.3 CXCR4/SDF-1 and HSPC trafficking

SDF-1 and its receptor, CXCR4, have been implicated in the attachment and retention of HSPC in the BM niches (section 1.6.6). In addition to this, the movement of HSPC into and out of the BM niches is largely dependent on the CXCR4/SDF-1 pathway (Lapidot & Petit, 2002; Lapidot et al., 2005). Migration, and mobilisation of HSPC, in both steady-state homeostasis and injury, are SDF-1/CXCR4 dependent (reviewed in Cottler-Fox et al., 2003).

Early in vitro studies described the chemotactic effect of SDF-1 on primary HSPC (Aiuti et al., 1997) and the MO7e haemopoietic cell line (Kim & Broxmeyer, 1998). This effect was later shown to be CXCR4 specific (Wright et al., 2002). SDF-1 in combination with vascular ligands, such as ICAM-1 and VCAM-1, mediate the firm (shear-resistant) adhesion of HSPC to the vessel wall before they transmigrate into the BM (Page et al., 1992; Springer, 1994;
Schweitzer et al, 1996; Butcher & Picker, 1996; Premack & Schall, 1996; Frenette et al, 1998). The impact of SDF-1 on these integrins was also shown to influence transendothelial migration of CB CD34+/CXCR4+ cells (Peled et al, 2000).

In addition to the integrins, additional migration pathways were later shown to be SDF-1 dependent. Avigdor and colleagues demonstrated that SDF-1–induced migration of HSPC is CD44 dependent (Avigdor et al, 2004). Moreover, in vivo, SDF-1 indirectly increases transendothelial migration by inducing secretion of the metalloproteinase MMP-9 by osteoclasts (Yu et al, 2003). MMP-9 then releases the normally membrane bound SCF into circulation (Heissig et al, 2002). The SDF-1 mediated osteoclast activation and cytokine (such as SCF) release in the BM induces quiescent stem cells to proliferate (Heissig et al, 2002) and facilitates HSPC migration (Lapidot & Petit, 2002).

Interestingly SDF-1 appears to specifically induce the transmigration of the more primitive stem cell subset of the population (Peled et al, 2000). This observation is particularly significant, as the migration of CD34+ cells to an SDF-1 gradient in vitro was shown to be analogous to their in vivo repopulation potential in transplanted patients (Voermans et al, 2001).

1.7.3.1 Modulations of the SDF-1/CXCR4 pathway

Because of its central roles in HSPC migration and retention the CXCR4/SDF-1 pathway was quickly utilised to enhance mobilisation or alter the behaviour of transplantable cells. SDF-1 pre-treatment was shown to promote proliferation of MPB CD34+ cells whilst sustaining their survival (Lataillade et al, 2000). In long-term culture of MPB CD34+ cells with cytokines, SDF-1 increased the percentage of primitive progenitors and subsequently clonogenicity was enhanced (Lataillade et al, 2000). Intravenous injection of SDF-1 significantly increased the levels of CB and
MPB CD34+ cell homing to the BM and spleen of immunodeficient mice (Dar et al., 2005).

Other molecules that have been utilised for the modulation of this pathway include several CXCR4 antagonists such as TN14003 (Liang et al., 2004), NefM1 (Bumpers et al., 2005) and MSX-122 (Liang et al., 2012). The most widely used CXCR4 antagonist, however, especially in haematology, is AMD3100 (Liles et al., 2003). With or without G-CSF co-administration, this molecule can enhance HSPC mobilisation in mice (Broxmeyer et al., 2005), dogs (Burroughs et al., 2005), monkeys (Larochelle et al., 2006) and humans (Liles et al., 2003; Devine et al., 2004; Hübel et al., 2004; Broxmeyer et al., 2005; Grignani et al., 2005; Liles et al., 2005; Larochelle et al., 2006) and has been approved by the FDA for stem cell mobilisation.

In the context of engraftment of transplanted stem cells much interest has centred on an indirect modulation of the CXCR4/SDF-1 pathway via the CD26 receptor. CD26 is a peptidase expressed on HSPC as well as on various stromal cell populations. It specifically targets and cleaves SDF-1, abrogating its chemotactic effect. The role of CD26, as well as the effects of its blockade on HSPC function, are discussed in Chapter 7.

1.7.4 Sympathetic nervous system (SNS) and HSPC trafficking

Early studies observed that the haemopoietic system is influenced by circadian rhythms (Goldeck, 1948). More specific data regarding the oscillations of several classes of haemopoietic progenitors (CFU-S, CFU-GM, BFU-E, CFU-E, and CFU-GEMM) in synchrony with circadian rhythms were later produced by several groups (Aardal & Laerum, 1983; Sletvold et al., 1988; Smaaland et al., 1992). Interestingly, the numbers of haemopoietic progenitors in the PB significantly increase after chemical sympathectomy (Maestroni, 1998). At the same time parallel observations were made for collagen synthesis and bone formation which are also circadian rhythm
dependent. Peak bone remodeling in mice, which is associated with HSC release from the BM, occurs during the environmental light period (Simmons & Nichols, 1966). This is related to the circadian fluctuations of a variety of hormones regulating skeletal muscle function such as parathyroid hormone (Fraser et al, 2004) and leptin (Fu et al, 2005).

Further expanding on these observations, D’Hondt and co-workers reported significant variations in the engraftment levels of HSPC in the BM, spleen and thymus when the donor animals had been exposed to light/dark cycle changes (D’Hondt et al, 2004). Finally, the Frenette group clearly demonstrated that murine HSPC have very specific circadian oscillations, with a peak PB concentration 5 hours after initiation of light, while the SDF-1 levels in the BM are inversely correlated, peaking when HSPC circulation reaches nadir (Lucas et al, 2008). The authors demonstrated that this was SNS dependent, as disruption of the system abrogated the circadian progenitor mobilisation (Méndez-Ferrer et al, 2008). More specifically they demonstrated that this decrease in SDF-1 levels in the BM is controlled by the \( \beta_3 \) adrenergic receptor which is expressed on BM stroma.

In the search for the connective link between the sympathetic nervous system (SNS) and HSPC mobilisation Schneider and co-workers demonstrated that the receptors for the mobilising agent G-CSF are expressed by neurons in the central nervous system (Schneider et al, 2005). Soon after, Katayama and colleagues reported, using mice with abnormal nerve conduction (Cgt\(^{-/-}\)), that G-CSF (or fucoidal) treatment fails to mobilise HSPC to the PB. Importantly they showed that the proteolytic activity of G-CSF in the BM is preserved, but the progenitors did not egress the BM space (Katayama et al, 2006). Surprisingly, SDF-1 levels in the BM of the Cgt\(^{-/-}\) mice are decreased after G-CSF administration which is in agreement with previous reports in normal mice (Lévesque et al, 2003). Bone expression of SDF-1, however, is not similarly altered. Additionally, osteoblast function as well as the adrenergic
tone were disrupted in the \textit{Cgt}^{−/−} mice (Katayama \textit{et al}, 2006). G-CSF acts on osteoblasts through norepinephrine signaling (Katayama \textit{et al}, 2006) while it also reduces reuptake of the neurotransmitter in the BM (Lucas \textit{et al}, 2012). Moreover, HSPC mobilisation is significantly enhanced after β2 agonist administration (even in norepinephrine deficient mice) (Katayama \textit{et al}, 2006). In the same context Spiegel and co-workers demonstrated that MPB CD34$^+$ cells migrate towards dopamine and norepinephrine \textit{in vitro} (Spiegel \textit{et al}, 2007).

A variety of nervous system associated receptors and peptides have been shown to affect the behaviour of HSPC. These interactions as well as their use to modulate HSC engraftment post-transplantation are discussed in detail in Chapter 8.

1.8 \textbf{HSPC expansion}

One of the critical factors that influence successful engraftment as well as timely haemopoietic reconstitution and recovery is the number of HSPC delivered to the recipient (Schwella \textit{et al}, 1995; Pérez-Simón \textit{et al}, 1998). The clinical application of HSPC transplantation may, therefore, be limited by HSC numbers, as discussed earlier (section 1.3.4). For this reason much effort has been directed at expanding these cells \textit{ex vivo}.

A protocol that enables the \textit{ex vivo} expansion of functional HSPC would also allow for the use of small grafts, such as umbilical CB grafts, or grafts from patients who have failed to mobilise an adequate number of cells due to previous chemo/radiotherapy. In addition to this, an efficient \textit{ex vivo} expansion protocol could also be used in gene therapy protocols, where the HSPC of the patient are harvested, therapeutic genes are transferred or defective genes replaced, accordingly and the cells are returned to the patient (reviewed in Verfaillie, 2002). In this approach, which is usually utilised in the treatment of immunodeficiency disorders, gene transfer requires the viral
transduction of a high number of cycling (post mitotic) HSPC (Lewis & Emerman, 1994). Lentiviral vectors are able to transduce both dividing and non-dividing cells, but for HSPC, progression to G₁ is still necessary (Sutton et al, 1999; Case et al, 1999).

1.8.1 Cytokine HSPC expansion

Ex vivo HSPC expansion strategies have largely employed varying combinations of cytokine cocktails. A list of haemopoietic cytokines and their stimulatory function is shown in Table 1.3. Exposure of HSPC to cytokines such as IL-3, IL-6, SCF, Flt3L and TPO in liquid cultures has been shown to induce entry into cell cycle and subsequent proliferation of HSPC. Efforts to achieve maximal benefit of the cytokine expanded HSPC have, however, been hampered by deleterious changes in the function of these cells, that are acquired in culture (reviewed in Srour et al, 1999; Dahlberg et al, 2011). Animal models of HSPC transplantation have demonstrated that this is related to the detrimental effects on the homing and engraftment of these cells to the BM of the recipients.

More specifically, exposure of HSPC to cytokines ex vivo has been shown to reduce their engraftment after infusion in murine homologous and xenogeneic transplantation models (Peters et al, 1996; Guenechea et al, 1999; Szilvassy et al, 2000; Young et al, 2001). Similar observations have been made on retrovirally transduced cells in the context of gene therapy (Dorrell et al, 2000; Demaison et al, 2000).

The first use of ex vivo cytokine-expanded MPB CD34⁺ cells in a clinical trial was in 1996 (Alcorn et al, 1996) where an 8-day expanded and an unmanipulated graft where co-injected to the patients. This was followed by a similar study using CB cells in 2002, when a non-expanded portion of the graft was transplanted to conditioned patients at day 0, followed by the expanded portion on day 10 (Shpall et al, 2002). This treatment achieved a 56-fold
increase of total cell number and 4-fold increase of CD34+ cells. Although, the expanded graft was well-received and no graft-related toxicities occurred, the time to neutrophil recovery (in comparison to non-expanded cells) was not improved.

Early studies investigated whether the engraftment defect of cytokine cultured cells is related to their inability to home to the haemopoietic niches effectively. Support for that hypothesis came from murine models where HSPC homing to the BM or the spleen of the recipient animals was reduced after exposure to cytokines ex vivo (Szilvassy et al, 1999; van der Loo & Ploemacher, 1995). These results were later confirmed in xenogeneic transplantation systems, using human, cultured HSPC. Previous work in our laboratory demonstrated that following culture in cytokines MPB HSPC have significantly reduced homing to the BM and spleen of NOD/SCID mice, whereas large portions of the transplanted cells are trapped in the lungs of the recipients (Ahmed et al, 2004).

The inability of the ex vivo expanded cells to egress from the circulation into haemopoietic tissue could be related to a change in the ability of these cells to permeate the BM endothelial cell barrier. However, previous work in our laboratory studied the process of transendothelial migration separately in in vitro assays and found cytokine activated cells to readily adhere to and migrate through the endothelial surface (Yong et al, 1998). Although these assays do not accurately mimic the in vivo homing process, which involves circulation, recognition and extravasation through BM vascular endothelium and migration into the haemopoietic microenvironment, they do indicate that cytokine activation increases the mobility of the HSPC and it certainly does not impair their ability to migrate across vascular endothelium.

Clinical trials using cytokine expansion of grafts are on-going but these data have prevented the general use of expanded HSPC in the clinic.
Subsequently, research has been redirected to identifying and modulating the aspects that are responsible for the detrimental effect of cytokine exposure on HSPC engraftment.

1.8.2 Alternative HSPC expansion protocols

In the last decade, a lot of the related research has focused on identifying alternative methods for *ex vivo* expansion of HSPC, usually in combination with cytokines. Initial efforts included the overexpression of the P glycoprotein pump genes *MDR1* and *ABCG2* (Bunting *et al*, 1998, 2000) and a retinoic acid receptor agonist (ATRA) (Purton *et al*, 2000) but the expansion was comparatively small with long culture times.

1.8.2.1 Notch signalling cascade

A key pathway that has been used for the expansion of HSPC (both *ex vivo* and *in vivo*) is the Notch signaling cascade. Notch is a “single-pass” transmembrane receptor involved in proliferation signaling, whereas inhibition of it is associated with differentiation. Activation of the Notch pathway maintained HSPC in an undifferentiated state while self-renewal was stimulated (Stier *et al*, 2002; Suzuki *et al*, 2006). These results were recently tested in a clinical setting where expansion of CB CD34+ cells in the presence of Notch ligand (Delta-1) enhanced engraftment of the transplanted cells leading to faster neutrophil recovery (Delaney *et al*, 2010).

1.8.2.2 Wnt signaling

Another important pathway in HSPC expansion is Wnt signalling. Activation of this pathway was shown to directly regulate HSPC proliferation (Reya *et al*, 2003). More specifically, overexpression of activated β-catenin increased HSPC numbers while Wnt signaling inhibition prevented growth (reviewed Malhotra & Kincade, 2009). Based on these results the authors used a chemical inhibitor of GSK-3β, 6-bromoindirubin 3′-oxime (BIO), which delayed
cell cycle progression by increasing cell cycle time. They used this inhibitor on human HSPC cultured with SCF, TPO and Flt3L for 24 hours and demonstrated increased cellularity and engraftment in recipient mice (Ko et al, 2011).

1.8.2.3 Transcription factors

Homeobox protein-4 (HOXB4) is a sequence-specific transcription factor that has a role in development. In 1995 the laboratory of K. Humphries showed that engineered overexpression of HOXB4 induces proliferation of HSPC in vivo (Sauvageau et al, 1995). Seven years later the same group showed that using the same strategy they could achieve expansion of HSPC ex vivo, more than 3 logs higher than the untreated controls in short-term liquid cultures (Antonchuk et al, 2001). In a similar manner, induced expression of another transcription factor (SALL4) was more recently shown to expand CD34+/CD38− cells by more than 10,000-fold, in long-term cultures with cytokines (Aguila et al, 2011). SALL4 is a zinc-finger transcriptional factor with an important role in the pluripotency of embryonic stem cells, in association with Oct4 and Nanog, and is constitutively expressed in leukaemia cell lines and primary cells.

1.8.2.4 Epigenetic mechanisms

A number of groups studying HSPC expansion have reported encouraging results by manipulation of epigenetic mechanisms. Such compounds include valproic acid, which increases histone H4 acetylation of the HOXB4 and AC133 sites (De Felice et al, 2005), chromatin- modifying agents (Araki et al, 2009) and a histone acetyltransferase inhibitor (Garcinol) (Nishino et al, 2011). The latest successful attempt at HSPC expansion utilising epigenetic mechanisms is the addition of nicotinamide (a form of vitamin B3) to liquid cytokine cultures. This treatment inhibits sirtuin 1, which is a nicotinamide adenine dinucleotide-dependent histone deacetylase. The authors describe
an expansion of the CD34+/CD38- population and increased engraftment levels (Peled et al, 2012).

### 1.8.2.5 Proteins, bioreactors and other HSPC expansion protocols

Protein treatments used for the ex vivo expansion of HSPC include sonic hedgehog (a morphogen with multiple roles in development) (Bhardwaj et al, 2001), angiopoietin (Arai et al, 2004; Zhang et al, 2008), serotonin (Yang et al, 2007) and pleiotrophin (a neurite outgrowth factor with high affinity for heparin) (Himburg et al, 2010).

In 2003, Jaroscak and colleagues performed the first clinical trial in which the HSPC were expanded in a bioreactor (Aastrom Biosciences) for 12 days but no improvement was observed in the times to myeloid and platelet recovery (Jaroscak et al, 2003).

Aldehyde dehydrogenase is required for the conversion of vitamin A to retinoic acids. Inhibition of this enzyme results in 3.4-fold expansion of human repopulating cells (Chute et al, 2006).

Cellular copper has been shown to be involved in the proliferation and differentiation of HSPC. Addition of a copper chelator in liquid cultures of HSPC expanded the progenitor population while retaining their engraftment potential (Peled et al, 2004, 2005). Based on these observations a phase I/II trial was performed to test the safety and engraftment levels of cytokine expanded CB CD133+ cells pre-treated with the copper chelator (De Lima et al, 2008).

In an alternative strategy, a clinical trial is being conducted at the University of Texas MD Anderson Cancer Center, in which CB HSPC are cultured with related donor mesenchymal stem cells (and cytokines) for 14 days, achieving 14-fold expansion of the CD34+ fold population, with no graft-related toxicities (reviewed in Robinson et al, 2011). The co-culture system, however, is
practically difficult to translate to the clinic, mainly due to the complexities of introducing mixed cell populations in the patients.

1.8.2.6 Small molecule compounds

In the last few years the discovery of small molecule compounds has attracted a lot of interest in HSPC expansion. One example is NR-101, an MPL agonist which was able to induce 2.9-fold increase of the CD34⁺ subpopulation in a 7-day culture (Nishino et al, 2009). Two other small molecules (zVADfmk and zLLYfmk) induced a 30 to 70-fold expansion of the total cell number when added in culture with cytokines for 10 days with a 3-fold increase in the CD34⁺/CD38⁻ subgroup (Sangeetha et al, 2010).

An exciting, recent discovery in this field, is that of StemRegenin 1 (SR1), an aryl hydrocarbon receptor antagonist, which was shown to aid self-renewal of HSC by inhibiting the aryl hydrocarbon receptor (Boitano et al, 2010). Culture of HSPC with SR1 (and cytokines) increased the CD34⁺ cell population by 50-fold, whereas the increase in repopulating cells was 17-fold, compared to cytokines alone. These results were recently confirmed in an automated culture system (Csaszar et al, 2012).

Despite much advancement in the search for ex vivo HSPC expansion protocols none of the described strategies have been successfully translated to the clinical practice so far. Moreover, almost all of the described treatments include cytokine cocktails to induce proliferation. Exposure of HSPC to cytokines is also essential for the viral transduction required for gene therapy. For these reasons it is important to understand the molecular basis for the observed loss of homing and engraftment resulting from the ex vivo culture with cytokines.
1.9 PhD aims

The initial aim of this work was to dissect and describe the changes in engraftment after culture of MPB CD34+ cells in cytokines. To achieve this, short-term homing, BM attachment and long-term engraftment of fresh and cultured HSPC were separately studied using \textit{in vitro}, \textit{ex vivo} and \textit{in vivo} assays (Chapters 3, 4, 5 and 6).

Based on these results, the second aim of this thesis was to explore strategies to pre-treat the cultured cells \textit{ex vivo} so as to modulate and improve their function post-transplantation \textit{in vivo}. Two separate pathways were exploited in this attempt; Firstly, CD26 inhibition has been demonstrated to improve long-term engraftment of fresh HSPC due to increased SDF-1 availability. Here CD26 inhibition was used to modulate the behaviour of cultured MPB HSPC (Chapter 7). Secondly, increasing evidence supports an important regulatory role of the sympathetic nervous system in haemopoiesis. The cytokine-induced changes in the expression and function of neurotransmitter receptors on HSPC were studied. Finally, neurotransmitter pre-treatment of MPB HSPC was tested for its effect on their adhesion and migration \textit{in vitro}, as well as their homing and engraftment \textit{in vivo} (Chapter 8).
2 General Methods

2.1 HSPC cell isolation, culture and characterisation

2.1.1 Primary haemopoietic stem cell source

MPB was obtained from patients and donors at University College London Hospitals (UCLH) after informed consent, in accordance with UCL/UCLH Committee on the Ethics of Human Research. HSPC were harvested from healthy individuals, donating for allogeneic HSCT, after a 5-day treatment with G-CSF (10µg/kg, daily). HSPC were also collected from lymphoma and myeloma patients on the University College London Hospitals NHS Trust stem cell programme. In this case the HSPC were collected after mobilisation with G-CSF and cyclophosphamide or ESHAP (a regimen containing cisplatin, etoposide, cytarabine and methylprednisolone), (Velasquez et al, 1994; Watts et al, 2000).

2.1.2 CD34+ cell isolation

CD34+ cells were isolated using a large scale CliniMACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with antibodies recognising the “QBEnd/10” CD34 epitope in accordance with manufacturer’s instructions as described in (Watts et al, 2002). The large scale selections were performed by Dr MJ Watts and Mr SJ Ings and the surplus to the clinical requirements was donated for this research.

CD34+ cells were also immunomagnetically selected using the VarioMACS LS column (Miltenyi Biotec). Cells from 1-2ml of MPB stem cell harvest product were washed once in 10ml of 10% Ficoll-Paque Plus (GE Healthcare, Chalfont St Giles, UK) in PBS (PAA, Pasching, Austria) solution, pelleted by centrifugation (1200rpm for 10 minutes). The pellet was then resuspended in 10ml PBS (PAA) added drop-wise and centrifuged at 1600rpm for 10 minutes.
and then, following aspiration of the supernatant, resuspended in 100 µl of anti-CD34 iron/dextran conjugated antibodies (Miltenyi Biotec) and incubated for 40 minutes at 4°C with regular resuspensions. The VarioMACS LS column, placed in its magnetic retainer, was washed once with 3ml of CliniMACS (PBS/EDTA buffer, Miltenyi) to remove residual azide. The immunolabelled cells were then washed once in 10ml CliniMACS (Miltenyi) and the cell pellet resuspended in 1ml of CliniMACS and loaded onto the column. Retained CD34⁺ cells were washed once with 3ml of CliniMACS 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. Both CD34⁺ cell preparations (CliniMACS and VarioMACS) had an average purity of 98±1%, as evaluated by immunofluorescence.

2.1.3 Cell culture

CD34⁺ selected cells were cultured in X-VIVO 10 (BioWhittaker, Walkersville, MD) with 1% human serum albumin (HSA) (Zenalb 20) and supplemented with SCF, FLT-3 (50 ng/ml), IL-3 and IL-6 (20 ng/ml). They were seeded at a density of 0.25-1x10⁶ cells/ml and incubated at 37°C and 5% CO₂. Unless otherwise stated, the cells were cultured for 72 hours under these conditions. After this period CD34 percentage was 94±2% and viability was 71±9%.

2.1.4 Cytometry

The number and viability of all the cell populations used were measured prior to every experiment with a haemocytometer or by FACS analysis.
2.1.4.1 Cell measurement by haemocytometer

To measure cell number and viability with a haemocytometer, an appropriate dilution of the sample was produced in 0.4% Trypan Blue solution (Sigma). The viable cells, not stained by Trypan Blue, were scored.

2.1.4.2 Cell measurement by FACS

In some experiments the cell numbers and viability were assessed by FACS analysis. CaliBRITE Beads (Beckman Coulter, CA, USA) were washed in PBS/10% BSA and resuspended in the same solution at a concentration of $1 \times 10^6$/ml. An appropriate volume was mixed with the cells and the sample was analysed on the flow cytometer. The number of cells/bead was established and based on that, the total cell number could be calculated. To measure the viability, a bivariate dot plot of narrow angle versus wide angle scatter was produced and non-viable cells were identified by virtue of their increased wide angle scatter and decreased narrow angle scatter.

2.1.5 Freezing and thawing of CD34$^+$ cells

When more than the required number of CD34$^+$ cells was isolated the residual cells were frozen. After washing, the pellet was resuspended in FCS and chilled for 30 minutes on ice before equal volume of FCS/20% DMSO was slowly added. The cells in the final solution of FCS/10% DMSO were stored in liquid nitrogen.

To thaw the frozen cells, the cryovial was briefly placed in a waterbath (37°C). The cells were then resuspended in 10ml warm HBSS/10% FCS and spun at 1,600 rpm for 10 minutes. Freshly isolated vs. cryopreserved cells have been compared for CFC output, in vivo homing and engraftment, and no significant differences were found between the two.
2.1.6 Cell cycle analysis

CD34+ cells (0.5-1x10^5/tube) were pelleted and resuspended in 50µl PBS, to which 450µl of ice-cold 70% methanol was added. The cells were then washed again (in PBS) and incubated in 500µl of staining solution with propidium iodide (section I.10) at 37˚C for 30 minutes. The cell suspension was then directly analysed on the flow cytometer.

2.1.7 Immunophenotyping

To determine expression of surface antigens on HSPC, aliquots of 5x10^4 cells were incubated with saturating concentrations (5-20µl) of either specific FITC, PE, PerCp, PECy5 and APC conjugated antibodies or the appropriate matching isotype control antibodies, in 50µl of PBS/0.1% BSA, for 40 minutes on ice. Cells were then washed once, in 2ml PBS/0.1% BSA and fixed in 200µl of 2% paraformaldehyde or resuspended in PBS and analysed immediately. Samples were stored at 4°C in the dark until ready to run on the flow cytometer (Beckman-Coulter).

2.1.8 Clonogenic assays

Clonogenic assays were performed routinely as readout for most of the in vitro and in vivo assays, to assess the CFC activity. A proportion of the cells was added to a semi-solid methylcellulose based media (Methocult H4230 StemCell Technologies, Canada), supplemented with IL-3 (30ng/ml), SCF (10ng/ml), EPO (2U/ml), G-CSF (25ng/ml) and GM-CSF (25ng/ml), to support colony growth. An aliquot of each sample to be assessed was seeded in 2.5ml of media and was cultured in 0.5ml aliquots in 4 wells of a 24 well tissue culture plate at 37˚C and 5% CO₂. Colonies were counted 14 days later by morphologic criteria (if cell numbers/colony exceeded 64) with an inverted stereo microscope (Olympus Optical, London). Appropriate controls were set up to control for the variability in donor clonogenicity.
2.1.9 Long-Term Colony-Initiating Cell (LTC-IC) assays

LTC-IC assays were used in some experiments to study the behaviour of HSC, as this assay is the closest *in vitro* representation of the *in vivo* repopulating cells. These assays were performed on 48-well plates where 70,000 MS-5 cells in 250µl of MyeloCult (StemCell Technologies) were seeded per well. Gelatin was used to coat the wells prior to seeding of the MS-5 cells, to support attachment of the feeder layer. CD34+ cells resuspended in 250µl of MyeloCult were then added in each well. The plates were cultured at 37°C and 5% CO₂ for 5 weeks until “cobblestones areas” were formed, indicating presence of stem cells (Neben *et al.*, 1993). Half-medium changes with fresh MyeloCult (with hydrocortisone at 10⁻⁶ M) were performed weekly during the five weeks. After this period the HSPC and stroma were harvested from each well with a Trypsin/EDTA wash (Sigma, MO, USA) and a proportion was seeded in the methylcellulose media described above. The LTC-IC number in the sample was determined by the number of colonies produced two weeks later from this colony assay.

2.1.10 Quantitative-Polymerase Chain Reaction (Q-PCR)

For the Q-PCR analysis, RNA was extracted using the RNeasy Micro Kit according to manufacturer’s instructions (Qiagen, Hilden, Germany), and treated with DNase. Reverse transcription (RT) was carried out using the SuperScript III kit (Invitrogen) according to manufacturer’s instructions. For the Q-PCR reaction, SYBR Green master mix reagent (Sigma) was used and the amplification was done using the Mastercycler ep Realplex (Eppendorf). To avoid amplification of contaminating DNA and unprocessed mRNA, primers were designed to anneal the end parts of two exons, where possible. The program “Primer3” was used to design the primers with “GC” content between 40-60% and maximum size of 22 base pairs. GAPDH and RPL13A transcripts were used to normalise the data. Primers used are shown in Table
<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>F: 5’- GGGAAGGTGAAGTGGAGGT-3’&lt;br&gt;R: 5’- GGGTCATTGATGGCAACAAATA-3’</td>
<td>58</td>
</tr>
<tr>
<td><strong>RPL13A</strong></td>
<td>F: 5’- CCTGGAGGAAGAGAGGAAGAGA-3’&lt;br&gt;R: 5’- TTGAAGGACCTCTGTTGATTGTTGCAA-3’</td>
<td>60</td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td>F: 5’- CCCAGATGGAGAAAGCTCTG-3’&lt;br&gt;R: 5’- TACACCCCTGTGTTTGTGTC-3’</td>
<td>59.2</td>
</tr>
<tr>
<td><strong>VLA4</strong></td>
<td>F: 5’- AGACGTGCGAAGCAGCTCC-3’&lt;br&gt;R: 5’- TGGCTGTCTGGAAAGTGTGA-3’</td>
<td>59.2</td>
</tr>
<tr>
<td><strong>CXCR4 (1)</strong></td>
<td>F: 5’- CCTATGCAAGGAAGATCCATGT-3’&lt;br&gt;R: 5’- GGTAAGGCTCCAGACTGTGAC-3’</td>
<td>60</td>
</tr>
<tr>
<td><strong>CXCR4 (2)</strong></td>
<td>F: 5’- CAGCAGGTAGCAAAGGTAGGC-3’&lt;br&gt;R: 5’- ATAGTCCCCCTGAGCCATTT-3’</td>
<td>57</td>
</tr>
<tr>
<td><strong>NCADHERIN (1)</strong></td>
<td>F: 5’- CCATCAAGCCTGTGAGGTAC-3’&lt;br&gt;R: 5’- GCAGATCGGAACGGATAGCT-3’</td>
<td>59</td>
</tr>
<tr>
<td><strong>NCADHERIN (2)</strong></td>
<td>F: 5’- GTGCAAGAGAGACACTCT-3’&lt;br&gt;R: 5’- CCACCTAAATCTGCAGGC-3’</td>
<td>59.2</td>
</tr>
</tbody>
</table>

**Table 2.1: Q-PCR primer sequences.** The sequences and annealing temperatures for all transcripts analysed by Q-PCR.
2.1. Transcript expression in cytokine cultured cells is expressed as fold increase in expression, as compared to the levels detected in fresh cells.

1.9.1.1 Gel electrophoresis

The specificity of the Q-PCR products was verified by running them on a 2% agarose gel to confirm their size and by using the dissociation curve software of the Q-PCR system. For the electrophoresis, agarose was dissolved in TBE by heating in a microwave oven. Once cooled slightly, the ethidium bromide was added and the gel was poured into a mould. After the gel was set, the running buffer was added to cover the gel. The samples (mixed with loading buffer) were loaded onto the wells of the gel and were electrophoresed at 70mA until they were sufficiently separated. Detection of the ethidium bromide fluorescence (intercalated in DNA), was performed under UV illumination.

2.2 In vitro functional assays

2.2.1 Adhesion assays

Adhesion assays were performed on non-tissue culture treated 48-well plates (VWR, Leicestershire, U.K.). Plates were coated overnight at 4°C with ligands (N-cadherin 10µg/ml, OPN 5µg/ml, VCAM-1 10µg/ml, fibronectin 50µg/ml), blocked with PBS/1% BSA before incubation with hCD34+ cells (70,000 cells/150µl/well) in IMDM, 2% FCS at 37°C for 90 minutes. Non-adherent cells were removed with 3 washes of 300µl each and a proportion was seeded in methylcellulose for CFC quantification. Colonies were counted 14 days later, and percent progenitor adhesion was calculated based on the CFC output of the starting hCD34+ cell population. Where different conditions were tested, separate controls were set up for each condition.
2.2.1.1 LTC-IC adhesion

To measure LTC-IC adhesion, the assays were performed as described above. After the non-adherent cells were washed off, 70,000 MS-5 cells/250µl were added in each well, over the adherent hCD34+ cells. 10,000 cells (150µl) of the starting population of hCD34+ cells were seeded in a well, followed by 70,000 MS-5 cells, as a control. The LTC-IC cultures were maintained in Myelocult (StemCell technologies) as described above. Cobblestone area formation was assessed 5 weeks later. The cells were then transferred to methylcellulose and colonies were scored two weeks later. The number of colonies produced in comparison to the control wells indicated the percentage of adherent LTC-IC.

2.2.2 Migration assays

Migration assays were performed mainly through fibronectin (50µg/ml) coated membranes. In a few experiments VCAM-1 (10µg/ml) coated membranes were used. Transwell filters (3.0µm/6.5mm, 24-well plates, Corning) were coated with fibronectin/VCAM-1 at 4°C, overnight. The surplus of the coating solution was removed and the filter was allowed to dry before 600µl of IMDM/20% FCS was added with or without SDF (100ng/ml) at the bottom chamber of the well. Finally, 100,000 hCD34+ cells/filter (in 100µl) were added on the top chamber. The cells were allowed to migrate for 5 hours at 37°C, 5% CO₂. Clonogenic assays were carried out on an aliquot of the migrated cells from the bottom chamber, and percent progenitor migration was calculated using the CFC output of the original cell suspension.

2.2.2.1 LTC-IC migration

To determine LTC-IC migration, the assays were performed as described above. After the assay was completed, two dilutions of the migrated cells (1/6 and 1/12) were seeded in 48-well plates, with 70,000 MS-5 per well, in a final
volume of 500 µl, in Myelocult. Gelatin was used to help the attachment of the stromal cells onto the plates. As a control 10,000 cells of the initial hCD34+ population were seeded with 70,000 MS-5 cells. Quantification of the migrated LTC-IC was measured from the colonies produced 7 weeks later, in comparison to the control cultures, as described in section 2.1.9.

### 2.3 Animal models

#### 2.3.1 Animal details and maintenance

All procedures involving the use and care of animals were performed in accordance with the Animal Scientific Procedures Act (1986) and licensed by the Home Office. NOD/LtSz-PrKdcscid (Non-obese/ Severe Combined Immunodeficient-NOD/SCID) and β2 microglobulin null/NOD/SCID (β2m/NOD/SCID) mice were housed in specific pathogen free conditions, under positive pressure, in a “Venti Rack”, in individually ventilated cages (Biozone, UK and Techniplast, Italy) and supplied with sterile food, water and bedding. Sprague-Dawley rats were housed in open cages (3 animals per cage). All animals were housed in rooms maintained at 22°C, with an alternating 12-h light–dark cycle. Food and water were available ad libitum.

#### 2.3.2 Bone excision

All animals were sacrificed by exposure to increasing CO2 concentrations. Death was ascertained by cervical dislocation. The animals were then placed on their back and their fur was sterilised with a solution of 70% IMS, to decrease the possibility of contamination of the subsequent procedures and cell preparations. Using sterile curved dissecting scissors (Sigma), a small slit was cut in the fur, on the lower chest area. The peritoneal membrane was retained intact. The skin was then peeled down so as to expose the hind limbs. The femur was firmly grasped with sterile forceps (Sigma) and using bone cutters (S Murray & Co, UK) or dissecting scissors, the bone was cut
off, close to the hip joint. The foot was separated from the tibia (and fibula) at the tarsal joint. Finally the knee joint was cut in the centre. Ligaments and excess tissue were removed and the clean bones were placed into tubes with transfer medium RPMI/10% FCS with 20U/ml heparin (section I.1) until they were further processed.

2.3.3 Bone marrow preparations

The femurs and tibiae were removed, as described above and BM cell suspensions were prepared from them. The BM was thoroughly flushed from both sides of the bone with HBSS using a 5ml syringe (Terumo, Tokyo, Japan) with a 27 gauge needle (BD, NJ, USA). The BM from all four bones of the animal was collected in a six-well plate well and was passed through a 19 gauge needle (BD) to produce a single-cell suspension. It was then put through a 40µM cell strainer (BD) and collected into a 50ml polypropylene tube (BD) containing 0.5ml of FCS in a total volume of approximately 25ml. The cell suspension was centrifuged at 1,600rpm for 5 minutes and the pellet was resuspended and kept on ice for at least 10 minutes before the red blood cells were lysed. This was done with red blood cell lysis buffer (section I.10) containing 155mM NH₄Cl, 20mM NaHCO₃, and 1mM EDTA, which was prepared, filtered and used ice cold. To each pellet 2ml of the red blood cell lysis buffer was added and the cells were immediately placed on ice for 4 minutes. After that, the lysis was stopped with freshly prepared and filtered rescue buffer containing BSA and Glucose (section I.10), 10ml of which was added to each tube. The cells were centrifuged at 1,400rpm for 5 minutes and then washed again in 7ml HBSS/5% FCS (1,400rpm, 5 minutes). Finally the pellets were resuspended in HBSS/5% FCS and their volume, cell number and viability was counted by Trypan Blue staining, as described in section 2.1.4.1.
2.3.4 *In vivo* models

2.3.4.1 Homing assays

The aim of homing assays is to assess the localisation of systemically infused HSPC into the BM. To test HSPC homing to the BM, hCD34^+^ cells were injected by tail vein into β2m/NOD/SCID animals (2x10^6^ cells /animal). The animal injections were performed by Dr. Michael Blundell, at ICH. The animals were sublethally irradiated (up to 24 hours) before injection (3 Gy, ^137^Caesium source). 2x10^6^ cells in 200µl HBSS were delivered per animal (no antibiotics were added to the medium). Controls from the starting population of the infused cells were seeded in methylcellulose medium (MethoCult, StemCell technologies), supplemented with GM-CSF 25ng/ml, SCF 10ng/ml and IL-3 30ng/ml (2,000 cells/2ml methylcellulose). These conditions are selective for growth of human colonies. This was confirmed in experiments in which no colony growth was seen when BM from saline-injected animals was seeded.

Animals were sacrificed 18-24 hours later and femurs and tibiae were obtained for analysis of homing of human CFC. BM preparations of the femurs and tibiae of the animals were produced as described in section 2.3.3. From the total number of cells harvested from each animal, 2x10^6^ (live) cells were seeded in the human specific methylcellulose described above. Colonies were scored 14 days later. Total human colonies per animal were calculated on the basis of the assumption that 2 tibiae and 2 femurs represent 16% of total BM (Chervenick *et al*, 1968) as shown below and were expressed as a percentage of the number of GM-CFC infused.

Calculation for % homing of GM-CFC to BM:

\[
H = \frac{A \times B \times (100/16) \times 100}{CxD}
\]
H= homing

A= Total number of BM mononuclear cells harvested (x10^6)

B= Colonies produced per 10^6 total BM mononuclear cells seeded

C= Colonies produced per 1000 cells from the starting hCD34+ population

D= hCD34+ cells infused (/1000)

The sex of the animals was found not to affect the levels of HSPC homing to the BM (Figure 2.1).

2.3.4.2 Engraftment assays

Engraftment assays represent the long-term retention and haemopoietic function of transplanted HSPC in the recipient’s haemopoietic tissue. Engraftment assays were performed on NOD/SCID or β2m/NOD/SCID mice aged 8 to 12 weeks, which were irradiated (3 Gy, 137Caesium source) up to 24 hours before the hCD34+ cells were infused. A dose of 2x10^6 cells/animal was delivered, unless stated otherwise. Both the cell dose and the radiation dose were optimised to produce the highest levels of engraftment for the β2m/NOD/SCID mice (Figure 2.2).

Eight to 10 weeks later, mice were sacrificed by CO2 inhalation and BM cells were flushed from femurs and tibiae. Following red cell lysis, cells were washed in PBS/0.1%BSA and 1x10^6 cells/tube were placed in 50µl of the same buffer for staining and flowcytometric analysis. The cells were stained with human-specific anti-CD19, anti-CD33 and anti-CD45 antibodies or their isotype controls (BD) for 40 minutes on ice. The samples were then washed in PBS/0.1% BSA, resuspended in 500µl PBS and analysed on a LSR (BD Biosciences) or a CyAn ADP (Beckman-Coulter) flow cytometer. Dead cells and debris were excluded using DAPI (4,6 diamidino-2-phenylinodole) staining and >150,000 DAPI-negative events were collected. Human engraftment was
Figure 2.1: The effect of the sex of the animals on homing. HCD34⁺ cells were injected IV to irradiated β₂m/NOD/SCID mice at a cell dose of 2x10⁶/animal. The levels of human HSPC homing to the BM were measured as described in section 2.3.4.1. No differences were observed between males and females in this context.
Figure 2.2: Irradiation and cell dose titration in engraftment. Freshly isolated hCD34+ cells were injected IV to irradiated β2m/NOD/SCID mice at cell doses of $2 \times 10^6$ and $5 \times 10^6$ per animal. The levels of human HSC engraftment to the BM were measured as described in section 2.3.4.2. *$p<0.05$, **$p<0.01$, Mann-Whitney test.
quantified as the proportion of live cells that were CD45+CD33+CD19− or CD45−CD33−CD19+ (Figure 2.3). An animal was considered to have successfully engrafted if the BM contained >1% hCD45+ cells.

2.3.4.2.1 IV delivery

IV delivery of hCD34+ cells to irradiated animals was performed with 0.5cc tuberculin, 27 gauge syringes (BD). The animals were placed in a restrainer (Harvard Apparatus, MA, USA) and 200µl of the cell suspension was infused in each animal via the tail vein. IV injections were performed by Prof. Dominique Bonnet, Dr. Fernando Afonso or Dr. Michael Blundell.

2.3.4.2.2 IB delivery

IB injections were performed by Prof. Dominique Bonnet and Dr. Fernando Afonso, using a 29 gauge needle (insulin syringe-BD). The hCD34+ cells (25µl) were inserted through the joint surface of the right tibia, into the BM cavity. This delivery method allows for separate analysis of engraftment of the injected bone versus the rest of the bones of the animal, thus giving information on both the retention and the ability of the transplanted cells to colonise other bones. Further details of this technique are provided in Chapter 4.

2.3.4.2.3 Animal sex and engraftment

Although not widely studied in terms of HSC engraftment in animal models, sex-associated hormones such as androgens and oestrogen have been shown to impact on HSC function post-transplantation (Igarashi et al, 2001; Goldberg et al, 2005, 2007). In the recently described γnull mouse, Notta and co-workers observed a significant difference between male and female animals in the levels of engraftment after human CB HSPC transplantation (Notta et al, 2010). In this work we found no sex-related differences in
Figure 2.3: Engraftment analysis by flowcytometry. The BM of the animals was flushed and the cells were stained with human-specific anti-CD19, anti-CD33 and anti-CD45 antibodies or their isotype controls (BD) for 40 minutes on ice. The samples were then analysed by FACS. The FITC isotype was used to set the gate for the CD45⁺ cells (top panel). The hCD45⁺ cells were quantified based on the gate set with the isotype control (middle panel). This population was then analysed for expression of hCD33 and hCD19 (bottom panel).
engraftment after IB delivery of MPB CD34+ cells in NOD/SCID mice (Figure 2.4). In contrast, engraftment in β2m/NOD/SCID mice after IV injection is significantly affected by the sex of the recipient animals (Figure 2.5). Male animals had engraftment of 1.794% (0.4162%-3.236%), following transplantation of 2x10^6 cells, whereas engraftment in females reached 25.65% (6.310%- 47.07%). Based on this observation, when β2m/NOD/SCID mice were used, the engraftment values were normalised to exclude the effect of the sex of the animals on the data and to reveal the real effect of the different treatments used. The raw data is also shown.

2.3.5 Ex vivo model

A novel ex vivo model was developed to represent the attachment of transplanted cells in the BM, using the long bones of 3-week old, male, Sprague Dawley rats. Development, optimisation, description and validation of this model are discussed in Chapter 5.

2.4 Statistical analysis

In the in vitro experiments, where the data demonstrated a Gaussian bell shaped distribution, the mean ± SEM is shown and for the statistical analysis paired or unpaired Students t-test were used (GraphPad Prism software, version 5), depending on whether the data was matched or not.

In the in vivo and ex vivo experiments, the data did not follow Gaussian distribution and thus non-parametric statistical analysis was performed. In that case the median (and range) is indicated and the statistical significance was determined by Mann-Whitney U Test (GraphPad Prism software, version 5) which compares un-matched groups. In both cases a p value of ≤ 0.05 was considered significant.
Figure 2.4: The effect of the sex of the animals on IB engraftment in NOD/SCID mice. Freshly isolated hCD34+ cells were injected IB to irradiated NOD/SCID mice at a cell dose of $5 \times 10^5$/animal. The levels of human HSC engraftment to the BM were measured as described in section 2.3.4.2. No differences were observed between males and females in this context.
Figure 2.5: The effect of the sex of the animals on IV engraftment in $\beta_2$NOD/SCID mice. HCD34$^+$ cells were injected IV to irradiated $\beta_2$ NOD/SCID mice at a cell dose of $2 \times 10^6$/animal. The levels of human HSC engraftment to the BM were measured as described in section 2.3.4.2. Engraftment is significantly lower in male animals. Statistical analysis by Mann-Whitney test, $p < 0.0001$. 

Figure 2.5: The effect of the sex of the animals on IV engraftment in $\beta_2$NOD/SCID mice. HCD34$^+$ cells were injected IV to irradiated $\beta_2$ NOD/SCID mice at a cell dose of $2 \times 10^6$/animal. The levels of human HSC engraftment to the BM were measured as described in section 2.3.4.2. Engraftment is significantly lower in male animals. Statistical analysis by Mann-Whitney test, $p < 0.0001$. 

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A: Effects of cytokine culture on MPB CD34+ cells

3 Effects of ex vivo culture on in vitro and in vivo functions of MPB CD34+ cells

3.1 Introduction

The use of cytokines is required in the majority of the ex vivo HSPC expansion protocols. Moreover, cytokine exposure is essential for the transduction of HSPC in gene therapy protocols. For many clinical applications MPB is the preferred cell source. For these reasons, it is important to study the cytokine-induced changes in the functional properties of MPB HSPC.

3.1.1 Choice of cytokines and rationale

Numerous cytokine cocktails have been assessed preclinically for their ability to improve gene transfer efficiency and to expand primitive HSPC while maintaining their repopulating capacity. The culture mix that has been used in this work contained SCF and Flt3L (at 50ng/ml) and IL-3 and IL-6 (at 20ng/ml) in X-VIVO 10 and 1% HSA. This specific cytokine cocktail has been validated by a number of different groups and has been shown to preserve the proliferation and pluripotency of haemopoietic stem cells (Conneally et al., 1997; Dao et al., 1997; Dao & Nolta, 1997; Bhatia et al., 1997b). This cytokine combination has also been validated by gene therapy protocols (Schilz et al., 2000; Demaison et al., 2000, 2002). A combination of cytokines including IL-3, IL-6, or IL-11 specifically, has been shown to be more beneficial for improving gene transfer efficiency and preserving the characteristic properties of target stem cells (Broudy, 1997; Gilliland & Griffin, 2002; Lyman & Jacobsen, 1998).
3.1.1.1 Stem Cell Factor (SCF)

SCF, also known as steel factor or c-Kit ligand, is a cytokine that plays a central role in the regulation and maintenance of haemopoiesis. It binds to the c-Kit receptor (CD117), which is expressed on primitive and mature haemopoietic cells (reviewed in Broudy, 1997). In terms of ex vivo expansion, SCF has been included in many effective cytokine combinations as it transduces signals essential for the proliferation of HSC and increases the production of erythroid, granulocyte-macrophage and megakaryocytic colonies (Brandt et al, 1992; Hoffman et al, 1993; Sui et al, 1995).

3.1.1.2 Fms-like tyrosine kinase receptor ligand (Flt3L)

Flt3L is a four helical bundle cytokine which stimulates tyrosine kinase activity leading to the expansion of haemopoietic cells (reviewed in Gilliland & Griffin, 2002). Binding of Flt3L to its receptor (Flt3), which is expressed on haemopoietic progenitors, promotes maintenance and proliferation of HSPC in in vitro cultures (Shah et al, 1996). When combined with TPO and SCF in particular, Flt3L is an “early acting” growth factor promoting self-renewal whilst stimulating the expansion of LTC-IC (Petzer et al, 1996).

3.1.1.3 Interleukin-3 (IL-3)

IL-3 is an activating interleukin with a broad target spectrum. It enables the differentiation of HSC into myeloid progenitors and stimulates the proliferation of all the myeloid cell types. Despite the fact that it is an early acting cytokine, that induces LTC-IC proliferation, there is some debate over its inclusion in stem cell expansion protocols as it also exhibits some features of late-acting growth factors. More specifically, it has been shown that IL-3 stimulation may decrease self-renewal and stem cell repopulation (Peters et al, 1996; Yonemura et al, 1996; Lu et al, 1996). Other groups, however, have shown retained engraftment potential of IL-3 expanded cells (Conneally et al, 1997;
Piacibello et al., 1999). It is not possible to directly compare these studies as they vary in terms of cell source, in vivo model and the use of serum free media and other cytokines. Nonetheless, cytokine combinations containing IL-3 remain the basis of many ex vivo expansion protocols.

3.1.1.4 Interleukin-6 (IL-6)

IL-6 is an interleukin with well described roles in inflammation and muscular biology that mediates its biological function through the gp130 subunit. Binding of IL-6 to its receptor (IL-6R) homodimerises gp130 which, in turn, induces intracellular signalling events. Although it does not have a role in haemopoietic cell expansion in vivo, the IL-6 receptor is expressed in committed progenitors but not by pluripotent stem cells (Tajima et al., 1996). Ex vivo, however, IL-6 has been included in many expansion protocols as an effective late-acting growth factor (Bernad et al., 1994; Shah et al., 1996; Moore et al., 1997).

Overall, the selection of cytokines used in this work combines both early and late acting molecules. This is an important characteristic of cocktails that are to be used for clinical transplantation, as a mere expansion of the primitive progenitors would render the patient neutropenic in the first instance. An appropriately balanced cocktail should include early acting growth factors, such as TPO, SCF, Flt3L and IL-3, to ensure the long-term engraftment of the infused cells in the recipient, as well as late acting ones, such as IL-6 and G-CSF (Table 1.3), which would stimulate a quick reconstitution of the haemopoietic system (Petzer et al., 1996; Herrera et al., 2001).

3.1.2 Chapter aims

This chapter describes the effect of cytokine culture (with IL-3, IL-6, SCF and Flt3L) on the properties and functions of MPB CD34+ cells. Specifically, the
effects on proliferation, cell cycling, surface receptor expression and migration in vitro, as well as on homing and engraftment in vivo, are examined.

3.2 Results

3.2.1 Effect of cytokine culture on proliferation and cell cycle status

Freshly isolated or thawed hCD34+ cells were cultured in X-VIVO 10 (a serum free medium suitable for cultivation of stem cells) with 1% HSA and supplemented with SCF and Flt3L (50 ng/ml) and IL-3 and IL-6 (20 ng/ml). HSPC cultured in this cytokine cocktail start expanding at 48 hours (1.29 ± 0.02-fold expansion, mean ± SEM) and reach 2.49 ± 0.07-fold increase at 72 hours (Figure 3.1). In the experiments described here the cells were used fresh or after 72 hours in culture unless otherwise stated.

During this culture period, the viability of the cells initially drops to a minimum of 63 ± 2%, mean ± SEM at 48 hours but increases to 82 ± 0.5% by 96 hours in culture (Figure 3.2). Fresh hCD34+ cells are mainly quiescent and so, the vast majority (95.46 ± 0.4%, mean ± SEM) are in G0/G1 phases of the cell cycle (Figure 3.3). Morphologically, at that stage they are small with scanty cytoplasm and high nuclear to cytoplasmic ratio. By 24 hours, as they begin to enter cell cycle, they start to increase in size and at 48 hours cytoplasmic basophilia can be detected as well as primary azurophilic granules and vacuoles. At 72 hours 34.9 ± 0.9% are cycling (Figure 3.3).

3.2.2 Effect of cytokine culture on clonogenicity and LTC-IC

To assess the effect of cytokine culture of MPB CD34+ cells on clonogenicity, 1,000 fresh and cultured cells were seeded in 2ml methylcellulose medium and the colonies produced were scored after 14 days. In Figure 3.4, total clonogenicity, as well as separate data for BFU-E and CFU-GM production
Figure 3.1: Proliferation of hCD34\(^+\) cells in cytokine culture. HCD34\(^+\) cells were cultured in X-VIVO with IL-3, IL-6, SCF and Flt3L for up to 96 hours. The cells were counted daily by CaliBRITE Beads beads or with a haemocytometer and the fold increase in cell number in comparison to the starting concentration is shown above for each timepoint. Mean ± SEM is indicated, n=14.
Figure 3.2: Viability of hCD34+ cells in cytokine culture. HCD34+ cells were cultured in X-VIVO with IL-3, IL-6, SCF and Flt3L for up to 96 hours. Viability was measured daily (morphologically) by FACS or by Trypan Blue exclusion. The percentage of viable cells for each timepoint is shown with mean ± SEM indicated, n=7.
Figure 3.3: Cell cycle status of hCD34+ cells in cytokine culture. HCD34+ cells were cultured in X-VIVO with IL-3, IL-6, SCF and Flt3L for 72 hours. The cell cycle status was analysed by Propidium Iodide (PI) staining. A) Representative histograms of PI staining before and after 72 hour culture in cytokines B) The percentage of cells in the G0/G1 phase, for 0, 24, 48 and 72 hours is shown with mean ± SEM indicated, n=5.
Figure 3.4: Clonogenicity of fresh and cultured hCD34+ cells. Fresh and (72 hours) cultured HSPC were seeded in methylcellulose (1000 cells/2ml of methylcellulose medium) and colonies were scored 14 days later. Total clonogenicity is shown on the top panel, CFU-GM production is shown in the middle panel and BFU-E in the bottom panel. Each donor is shown separately before and after culture with cytokines. Student's paired t-test,*p<0.05, **p<0.01, ***p<0.001, n=38.
are indicated for each donor (n=38). The clonogenic frequency after cytokine culture cells is higher compared to fresh cells, although the effect size is small. In more detail, mean total clonogenicity for fresh cells is 252.2 colonies/1000 cells and it increases to 311.2 colonies/1000 cells after 3 days of culture in cytokines (p<0.01, paired Student’s t-test). Mean CFU-GM production is 179.5 colonies/1000 cells for fresh cells and it increases to 206.1 colonies/1000 cells for cultured cells (p<0.05). Finally, average production of erythroid colonies from fresh cells is 70.96/1000 cells and it increases to 106.2 colonies/1000 cells after culture in cytokines (p<0.0001).

LTC-IC frequency of fresh and cultured cells was determined with the assay described in section 2.1.9. Briefly 1000 cells were seeded on mouse stroma and the HSC were harvested 5 weeks later and seeded in methylcellulose medium from which colonies were scored 2 weeks later. As shown in Figure 3.5, culture in cytokines does not appear to cause any significant difference in the frequency of LTC-IC produced in comparison to fresh hCD34+ cells. This observation, however, is based on a relatively small number of donors which may be insufficient to demonstrate small changes.

### 3.2.3 Effect of cytokine culture on CD34 and CXCR4 expression

Concomitant with the morphological changes observed on hCD34+ cells that are cultured with cytokines, the expression of specific surface antigens is altered. After 72 hours of culture in cytokines there is a small (5.78%) but statistically significant drop in the percentage of cells expressing the CD34 antigen (paired Student’s t-test, p<0.05) as shown in Figure 3.6.

Moreover CXCR4 expression also increases significantly when the HSPC are cultured in cytokines. As shown in Figure 3.7 A, 9.9 ± 2.2% (mean ± SEM) of fresh cells express CXCR4, increasing to 29.9 ± 4.3% after culture in cytokines for 72 hours (paired Student’s t-test, p<0.05). Increased surface
Figure 3.5: LTC-IC frequency of fresh and cultured hCD34+ cells. Fresh and (72 hours) cultured CD34+ cells were seeded in mouse stroma as described in General Methods and Long-Term Culture-Initiating Cells were scored 7 weeks later. The number of LTC-IC/1000 cells is shown separately for each donor before and after culture in cytokines. NS, Student’s paired t-test, n=6.
Figure 3.6: Timecourse of CD34 expression. A) Freshly isolated HSPC cells were stained for CD34 expression at 0, 16, 24, 48 and 72 hours of culture in the standard cytokine cocktail. The percentage positive cells are shown in the top panel and fold MFI difference (in comparison to isotype controls) is shown in the bottom panel. N = 6. B) MFI ratio is calculated as the Median Fluorescence Intensity of the stained cells divided by the Median Fluorescence Intensity of the isotype control (MFI2/MFI1)
Figure 3.7: Timecourse of CXCR4 expression. Freshly isolated HSPC were cultured in the standard cytokine cocktail for 72 hours and CXCR4 expression was assessed by FACS and Q-PCR. A & B) Surface protein expression of CXCR4 was assessed at 0, 24 and 72 hours and the mean percentage positive cells (A) and MFI ratio (B) are indicated. N=7. C) MRNA expression of CXCR4 in cytokine activated hCD34+ cells in comparison to fresh cells, as analysed by Q-PCR. Fold difference of receptor expression (black) in relation to fresh cells (white). Mean ± SEM, n=4.
protein expression is at least partly related to increased mRNA levels, as CXCR4 mRNA expression increases 4.1-fold after culture in cytokines (Figure 3.7 C).

Cytokine activation has similar effects on receptors related to adhesion and migration of HSPC, which will be discussed in Chapters 6 and 7.

3.2.4 Effect of cytokine culture on engraftment

In order to confirm that cytokine stimulation of human MPB CD34+ cells compromises engraftment potential as has been previously discussed, the engraftment of freshly isolated and cytokine activated hCD34+ cells in NOD/SCID animals was analysed by FACS analysis of murine BM performed at 8-10 weeks post transplantation (Figure 3.8). Freshly isolated CD34+ cells were stimulated with the standard cytokine mixture for 3 days and assessed for their engraftment potential, in comparison with fresh cells. NOD/SCID mice were used and 2x10^6 cells were delivered IV (tail vein) per animal. Human engraftment was quantified as the proportion of live cells that were CD45+CD33+CD19- or CD45+CD33-CD19+, as described in section 2.3.4.2. Animals that received fresh cells had a median engraftment of 3.05% (0.14%-23.3%) whereas animals that received cultured cells only achieved median engraftment levels of 0.47% (0%-3.87%, p<0.05, Mann-Whitney test).

3.2.5 Effect of cytokine culture on homing

The reduction in long-term engraftment of cultured HSPC has been partly attributed to the significant decrease in the levels of short-term homing of the transplanted cells to haemopoietic tissue, as has been described by this group (Ahmed et al, 2004) and others (Van der Loo & Ploemacher, 1995; Szilvassy et al, 2001). This defect is confirmed here, using sublethally irradiated β2m/NOD/SCID mice. 2x10^6 fresh or cultured hCD34 cells were delivered IV and short-term homing was assessed 18-24 hours later by
Figure 3.8: Cytokine culture reduces the long-term engraftment of hCD34+ cells. Fresh and cultured human MPB CD34+ cells were infused IV, by tail vein, into sublethally irradiated NOD/SCID mice at a dose of $2 \times 10^6$ cells per animal. Engraftment was analysed 8-10 weeks later by assessing expression of hCD45 with hCD33 or hCD19 in the BM of the recipient animals, by FACS. The levels of engraftment are shown separately for each animal with medians indicated. Statistical analysis by Mann-Whitney test, $p<0.05$. 
assaying the number of human CFC in murine BM, as described in section 2.3.4.1. Fresh cells achieve median levels of homing of 3.8% (1.1%-14.74%). After 72 hours of culture in cytokines, short-term homing of human CFC is reduced to 0.8% (0%-1.672%, p<0.0001, Mann-Whitney test, Figure 3.9).

3.2.6 Migration of fresh and cultured HSC

The transmigration of the donor HSPC from circulation through the sinusoidal endothelium to the haemopoietic tissue in the recipient is a prerequisite for their short-term homing and subsequently their long-term engraftment in the niches, as discussed in section 1.4.2. This trafficking of the HSPC is largely dependent on the SDF-1 gradient between the vessels and the microenvironment of the niche. Moreover, within the niches, the cells need to locate and attach to the appropriate components of the microenvironment such as the extra-cellular matrix proteins and stromal or osteoblastic cells. It has been previously shown by this group that transendothelial migration (through HUVEC cells) is increased following cytokine stimulation of MPB CD34+ cells (Yong et al., 1998). Here, the migration of fresh and cultured HSPC through fibronectin and VCAM-1 coated membranes was investigated. Both fibronectin (Vartio et al., 1987) and VCAM-1 (Simmons et al., 1992) are ligands highly expressed by BM stromal cells. These experiments aimed to establish whether intra-marrow migration of HSPC is defective after exposure to cytokines.

As is shown in Figure 3.10 both fresh and cultured progenitors significantly increase their migration through fibronectin and VCAM-1 in response to SDF-1 gradient. Moreover, as was previously observed with HUVEC coated membranes, culture of hCD34+ cells in cytokines increases baseline and SDF-1 directed progenitor migration for both ligands. SDF-1 directed migration of cultured cells reaches 29.82 ± 5.6% (mean ± SEM) for VCAM-1 and 44.17 ± 2.4% for fibronectin. Similar responses to SDF-1 and cytokine
Figure 3.9: Cytokine culture reduces the short-term homing of hCD34+ cells. Fresh and cultured human MPB CD34+ cells were infused IV (tail vein) into sublethally irradiated \( \beta_{2m}/\)NOD/SCID mice, at a dose of \( 2 \times 10^6 \) cells per animal. Short-term homing was determined by the percentage of human GM-CFC in the BM of the recipient animals, 24 hours after transplantation. The levels of homing are shown separately for each animal with medians indicated. Statistical analysis by Mann-Whitney test, \( p < 0.0001 \).
Figure 3.10: Migration of fresh and cultured HSPC. Migration of fresh and cytokine activated hCD34+ progenitors through VCAM-1 (top panel) or fibronectin (bottom panel) coated filters, as assessed by colony assays on migrated cells. SDF-1, at 100ng/ml, was placed in the bottom chamber of Transwells, where indicated. Each donor was tested in duplicate, mean ± SEM is shown, n=13 for fibronectin and 4 for VCAM-1. ***p<0.001, *p<0.05, paired Student’s t-test.
activation were observed for LTC-IC migration, although these primitive cells appear to be considerably less migratory than progenitors either before or after cytokine culture (Figure 3.11).

The effect of VLA-4 blockade on fresh and cultured progenitor migration towards an SDF-1 gradient was also tested. VLA-4 functions as the main receptor for both VCAM-1 and fibronectin on hCD34+ cells. As shown in Figure 3.12, blockade of VLA-4 completely abolishes migration of freshly isolated HSPC to SDF-1 through both fibronectin and VCAM-1 coated filters. After cytokine activation, however, blockade of VLA-4 has a lesser effect on migration across fibronectin, when compared with migration across VCAM-1. Isotype control experiments were performed to confirm the specificity of this effect on migration.

3.3 Discussion

BM reconstitution following HSC transplantation is a multi-step process in which the infused cells have to home to and lodge in the specialised niches of the BM microenvironment and this is followed by the onset of their proliferation (engraftment). Successful engraftment could be affected by variety of factors such as the cell cycle status, differentiation, clonogenicity and transmigration of the transplanted cells. The aim of this chapter was to study the effect of cytokine culture on these aspects of HSPC engraftment.

Culture of MPB CD34+ cells for 3 days in X-VIVO 10 with IL-3, IL-6, SCF and Flt3L achieved a 2.49-fold increase in cell number similar to the expansion described in CB by (Dorrell et al, 2000) and in MPB by (Danet et al, 2001) with only 17% loss of viability (by day 3). After exposure to cytokines HSPC quickly go into cycle with 35% of the cells cycling by day 3. The effect of the cell cycle status on long-term engraftment has been debated in the field by a number of groups. A number of studies have suggested that there is a loss of
Figure 3.11: Migration of fresh and cultured LTC-IC. Migration of fresh and cytokine cultured hCD34⁺ LTC-IC through fibronectin coated filters, as assessed by LTC-IC assays on migrated cells. SDF-1 at 100ng/ml was placed in the bottom chamber of Transwells where indicated. Each donor was tested in duplicate, mean ± SEM is shown, n=3. **p<0.001, *p<0.05, paired Student’s t-test.
Figure 3.12: VLA-4 blockade in migration assays. SDF-1 directed migration of fresh and cytokine activated hCD34+ progenitors through VCAM-1 (top panel) or fibronectin (bottom panel) coated filters, as assessed by colony assays on migrated cells. VLA-4 was blocked prior to migration, with a specific blocking antibody (5µg/ml for 40 minutes on ice), where indicated. The antibody was present during the assay. Each donor was tested in duplicate, n=4.
HSPC engraftment ability as cells progress into active phases of the cell cycle (Gothot et al., 1997; Glimm et al., 2000; Orschell-Traycoff et al., 2000; Szilvassy et al., 2000). Using highly purified murine stem cells Habibian and co-workers found this engraftment defect to be reversible, and to fluctuate as cells undergo synchronous cell cycle passage with minimum engraftment levels between late S and early G2 phases (Habibian et al., 1998). In human MPB, however, in our group we have been unable to rescue the defective homing (and hence engraftment) of cytokine-activated cells by manipulating cell cycle status. Although cytokine cultured, sorted G0/G1 cells had slightly higher short-term homing to the BM, compared with cells in S/G2/M, this was not statistically significant, and remained significantly lower than the levels seen with fresh cells (Ahmed et al., 2004). Importantly, sorted cells in different phases of the cell cycle (G0/G1 vs. S/G2/M) did not show differences in engraftment. These observations suggest that other factors apart from cell cycle status are likely to influence the engraftment capabilities of adult HSPC.

Another factor that could affect engraftment is the clonogenicity of the transplanted cells. Previous reports by (Danet et al., 2001) using MPB CD34+ cells from 4 donors cultured in a different cytokine mix (SCF, TPO and Flt3L) without serum, showed a rapid increase in the frequency of CFC in the first two days followed by a reduction on the third day to a frequency still higher than that of fresh cells. Here, the clonogenicity of MPB CD34+ cells after 3 days of culture was compared with that of fresh cells from 38 donors and a small but statistically significant increase was observed. More specifically, both CFU-GM and BFU-E as well as total clonogenicity increase after cytokine culture. The differences in clonogenicity between fresh and cultured cells as observed by us and Danet et al, however, could not explain the significant loss of engraftment after cytokine expansion of MPB CD34+ cells.
Similarly the LTC-IC content of expanded cells could be another factor affecting long-term engraftment and reconstitution of the recipient BM. In this context Danet et al report an increase of approximately 40 LTC-IC per 1000 cells after cytokine activation, in 4 donors (Danet et al, 2001). In this work, from 6 donors, there is no statistically significant difference in the frequency of LTC-IC produced from 1000 CD34\(^+\) cells before and after culture in cytokines. Although a higher number of donors is required to confirm these results, the data from both studies do not support a role of LTC-IC dilution as the underlying cause for the loss of engraftment of cultured HSPC.

Surface CD34 levels on cultured HSPC only decreased by 5% after 72 hours in cytokines, which suggests that, although there is some differentiation, as expected, the CD34 levels are too high to explain the profound loss of engraftment observed.

Another important receptor for homing, retention and engraftment of transplanted HSPC is CXCR4 (reviewed in Lapidot et al, 2005). Freshly isolated MPB CD34\(^+\) cells express relatively low levels of CXCR4 (9.9 ± 1.1%) but expression significantly increases after exposure to cytokines, reaching 29.87 ± 7.7% after 72 hours. Similar findings are reported by Peled and co-workers (Peled et al, 1999b). The importance of this receptor in engraftment has been underlined in experiments where blockade of CXCR4 on (CB, MPB and BM) HSPC prior to transplantation significantly reduced homing to the BM and long-term engraftment in NOD/SCID mice in vivo (Peled et al, 1999b; Lapidot & Kollet, 2002). Moreover, overexpression of CXCR4 on CB and MPB CD34\(^+\) cells increased the levels of homing to the spleen (but not the BM) of NOD/SCID mice by 2-fold (Kahn et al, 2004). In PB CD34\(^+\) cells CXCR4 overexpression has been associated with the most primitive subset of the population (Lataillade et al, 2000). In this context, however, despite the increased expression of CXCR4 after cytokine culture, the cells display reduced homing and long-term engraftment in the BM. This
suggests that the surface protein expression of CXCR4 alone does not
determine the homing behaviour of the cytokine cultured HSPC and other
factors or pathways are likely to influence their trafficking and retention in the
BM.

In summary, this chapter has described several aspects of HSPC biology that
are altered by cytokine stimulation, but no significant contributing factor to
impaired engraftment was identified. Short-term homing, which is essential for
retention and long-term engraftment of HSPC in the BM, is significantly
reduced after culture in cytokines. This observation is in agreement with
previous findings by (Ahmed et al, 2004). The defective homing observed is
not related to an inability of the cultured cells to cross the endothelial barrier
and enter the BM microenvironment. In fact, in migration assays through
HUVEC coated membranes (Yong et al, 1998) as well as through fibronectin
and VCAM-1 coated membranes (this work), cytokine activation significantly
increased random and SDF-1 directed progenitor migration. Additionally we
found this to be largely dependent on the VLA-4 receptor although
dependence on this receptor is reduced after cytokine culture. This is in
agreement with previous work by Foguenne and colleagues who showed that
engraftment of CB cells becomes less dependent on VLA-4 and more
dependent on VLA-5 after culture in cytokines (Foguenne et al, 2009). The
increased mobility of HSPC after cytokine exposure as observed in vitro,
could lead to loss of the transplanted cells to non-haemopoietic tissue in vivo
which we have previously postulated to contribute to reduced homing and
consequently engraftment.

Inefficient arrival of transplanted HSPC to the BM impairs the overall
engraftment therefore the next experiments were aimed at bypassing this
process by delivering HSPC directly into the BM of recipient animals.
4 Intra-bone (IB) delivery of HSC

4.1 Introduction

4.1.1 HSPC assays

In the murine system there is a long and successful history in the identification and description of HSC. Cell surface markers used to select HSC include Sca-1 and Thy-1 (Spangrude et al, 1988) as well as the LSK combination (Okada et al, 1992; Morrison & Weissman, 1994). Additional markers include CD34 (Osawa et al, 1996), Tie2 (Arai et al, 2004), EPCR (Balazs et al, 2006) and the “signaling lymphocyte activation molecule” (“SLAM”) protein family (Kiel et al, 2005) (discussed in section 1.2.1).

While these markers (or combinations of markers) can efficiently identify HSC in the murine system, only some of the information generated from the mouse models has been found to be applicable to human HSC and there is currently no definitive protocol to identify this population. For that reason a number of in vitro and in vivo approaches have been critical in describing the many facets of proliferation and differentiation of human HSPC (described in section 1.2.2).

4.1.2 Intravenous transplantation models

The NOD/SCID transplantation model has proven to be a reliable system for the detection of human haemopoietic-repopulating cells that are capable of multilineage differentiation and self-renewal (Laroche et al, 1996; Hogan et al, 1997; Cashman et al, 1997; Bhatia et al, 1998 and section 1.2.2.2.3). Moreover, in vivo assessment of engraftment is representative of stem cell transplantation in clinic and hence has additional translational value.
This assay, however, in which the infused cells are normally delivered by IV (tail vein or retro-orbital) injection, quite possibly underestimates human haemopoietic-repopulating cell frequencies. In these protocols, successful engraftment would require the circulation of the infused cells, the recognition and extravasation through BM vasculature and finally the migration and retention in a supportive haemopoietic microenvironment. In fact, a number of studies have indicated that the IV infused cells are sequestrated in the lungs and other organs with large capillary beds, like the liver, as they travel through the systemic circulation, and are lost (Cui et al, 1999; Wright et al, 2001; Jetmore et al, 2002; Wagers et al, 2002). As a result, only a small proportion succeeds in homing to the BM. The defective arrival of IV infused cells to the BM was first shown in syngeneic murine transplantation by Van der Loo and Ploemacher and was later confirmed using human CB in xenogeneic transplantation models (Van der Loo & Ploemacher, 1995; van Hennik et al, 1999; Cashman & Eaves, 2000). In agreement with these observations, in this work, we have shown that following tail vein injection, only 3.8% (1.110%-14.74%) of the infused MPB HSPC arrive in the BM of β2m/NOD/SCID mice (Figure 3.9).

Loss of the transplanted cells in non-haemopoietic tissue may cause some classes of HSC to remain undetected leading to underestimation of the overall engraftment levels. For these reasons a number of groups attempted to deliver the donor cells directly into the BM of the recipient animals thereby excluding the homing interference.

4.1.3 Transplantation models using IB injection

This delivery method was initially described by Kushida and co-workers, as a protocol to ameliorate autoimmunity in MRL/lpr mice (Kushida et al, 2001). It was soon, however, adopted by other groups to study the long-term engraftment of HSC. Two laboratories simultaneously published their results
on IB transplantation of human CB cells (Yahata et al., 2003; Wang et al., 2003). Yahata and co-workers focused on the advantages of this delivery method over IV infusion. They showed a 15-fold increase in repopulating cell frequency in the BM of NOD/SCID mice that had received CD34+/CD38− cells IB in comparison to IV delivery. They also performed secondary transplantation experiments where they showed significantly higher levels of engraftment from the mice that had initially received IB transplantation. Finally, they demonstrated multi-lineage differentiation and, using specific blocking antibodies, they showed that CXCR4 as well as VLA-4 and VLA-5 are essential for the long-term engraftment of IB transplanted cells (Yahata et al., 2003). A similar comparison between IV and IB transplantation of CB cells was performed a year later, by Castello and co-workers, using both the NOD/SCID and the C57BL/6J models. Similar to Yahata et al. they report a 15-fold increase in seeding efficiency in comparison to IV transplantation (Castello et al., 2004).

Wang and co-workers used the IB infusion protocol to better describe the lineage-negative/CD34− population of human CB cells. They showed that failure of these cells to engraft after IV injection was due to their defective homing, as when delivered IB they exhibited significantly higher levels of engraftment. They also studied the transmigration of these cells to other, non-injected, bones and found that the infused cells had successfully engrafted and differentiated in these bones, as well (Wang et al., 2003). Later that year, the Dick group used the same injection protocol to identify a highly repopulating group of human CB cells within the Lin−CD34+/CD38lo/CD36− population. They also demonstrated multi-lineage differentiation, faster kinetics (in comparison to IV delivery) and transmigration to non-injected bones (Mazurier et al., 2003).

As the advantages of IB transplantation in comparison to IV have been well described in these studies, this method was further used by those and other
researchers to investigate other aspects of stem cell behaviour. The Dick laboratory performed a direct comparison of xenogeneic transplantation models using IV and IB delivery methods and identified that IB transplantation of human CB cells was highest in anti-CD122–treated NOD/SCID mice (McKenzie et al., 2005). In a similar experimental set-up the same group later published on the advantages of the NSG and NOD/Shi-scid/IL2Rγnull mouse models in supporting CB engraftment (McDermott et al., 2010). The Bonnet laboratory used the same model to demonstrate that binding of fluorochrome-conjugated antibodies on the transplanted cells (for sorting purposes) significantly affects their long-term engraftment (Taussig et al., 2008). When the cells are infused IV there is peripheral clearance of antibody-coated cells by the spleen, whereas IB infusion allowed for better retention of the cells in the BM. In this context it was shown that the CD34+/CD38+ population, which was previously shown not to engraft through IV infusion (Bhatia et al., 1997a), achieves significant levels of engraftment when delivered intraosseously (Taussig et al., 2008).

4.1.4 Future prospects of IB transplantation

Experimentally, IB transplantation is a sensitive assay which improves the levels of homing and long-term engraftment and enables the direct investigation of the interaction of HSC with the BM. In addition to this it could be used to improve the results of transplantation in several settings such as when there is limited availability of HSPC or in experiments aiming to place stem cells of other lineages (CNS, muscle, etc.) in the BM. Clinically, the intraosseus injection protocol has been used since 1934 as a potential treatment of pernicious anaemia (Josefson, 1934) and later for the infusion of blood and fluids to critically ill patients (Tocantins et al., 1941; Spivey, 1987). In the context of BM transplantation, the first randomised trial between IB and IV infusion in humans was performed in the 1990s by Hägglund and colleagues in patients with haematological neoplasia (Hägglund et al., 1998).
They describe that, although intraosseous transplantation can be safely performed and it reduces the time of parenteral nutrition and antibiotics required, the overall haemopoietic recovery in comparison to IV was not improved. The high cell dose delivered in this trial, however, might have masked any possible differences. Generally the use of IB infusion in HSC transplantation is a protocol which, if optimised, could aid engraftment in situations where HSC number is limiting (such as with CB transplants or poorly MPB HSC samples).

4.1.5 Chapter aims

The major advantage of IB infusion is that it by-passes the homing process in which a large proportion of the infused cells are lost, even in populations that achieve comparatively high levels of long-term engraftment. This protocol, however, has not been tested with MPB progenitors. Therefore, the initial aim of this chapter was to test whether IB transplantation of MPB HSPC will increase engraftment in immunodeficient mice, compared to the IV delivery method.

Expansion of MPB CD34\(^+\) cells with cytokine cocktails results in a significant reduction of long-term engraftment (Chapter 3). As discussed in section 3.2.5, this is at least partly related to the inability of the expanded cells to home effectively to the BM of the recipient animals (Van der Loo & Ploemacher, 1995; Szilvassy et al, 2001; Ahmed et al, 2004). This was also confirmed in this work, as MPB CD34\(^+\) cells cultured in the presence of IL-3, IL-6, SCF and Flt3L, exhibited a 7-fold decrease in the average levels of short-term homing (Figure 3.9). For this reason, aim of this chapter was also to investigate whether direct intraosseous injection would rescue the levels of long-term engraftment of ex-vivo expanded MPB CD34\(^+\) cells.
4.2 Protocol

The IB transplantation experiments described here were performed in collaboration with Prof. Dominique Bonnet and Dr Fernando Afonso, at Cancer Research UK. Sample collection, cell isolation, cytokine activation (or any other ex vivo treatments described in following chapters) were performed at UCL. The cells were then transported on ice, to the Lincoln's Inn Fields Laboratories where the Bonnet group is based. Professor Bonnet or Dr Afonso then performed the IB injections in sublethally irradiated NOD/SCID mice. Aliquots containing a range of cell doses (1 to 25x10^5 cells) were prepared in (25µl) sterile HBSS.

The injection was performed using a 29-gauge needle. The mice were anaesthetised and the right knee was bent creating an inverted “V”. The needle was then inserted in the gap between the knee cap and the tibia through the top of the tibia and into the BM cavity. The cell suspension was then infused and the leg was rapidly placed in a horizontal position to close up the puncture site and prevent any leakage.

Assessment of engraftment levels was performed 8-10 weeks later by flow cytometry. The animals were sacrificed and the BM from the long bones and pelvis was flushed. In some experiments the BM of the injected right tibia was analysed separately from the rest of the bones. This protocol, in addition to the overall engraftment levels, allows for assessment of engraftment at the site of cell injection. Furthermore, the transmigration of the engrafted cells, from the site of injection to other haemopoietic tissue, can be studied. The flushed cells were stained with antibodies to hCD45, hCD33 and hCD19 and analysed on a MoFlow XPD flowcytometer. Human engraftment was quantified as the proportion of live cells that were CD45^+CD33^+CD19^- or CD45^-CD33^-CD19^+.
4.3 Results

4.3.1 IV vs. IB infusion

As previously discussed IB injection has been demonstrated to improve efficiency of engraftment using CB (Yahata et al., 2003; Wang et al., 2003), but similar data are lacking for adult MPB hCD34+ cells. Initial experiments here investigated the levels of engraftment of a series of MPB CD34-selected samples in irradiated NOD/SCID animals, comparing IB with IV (tail vein) injection using different cell doses. As seen in Figure 4.1, IB delivery led to higher levels of engraftment at all cell doses tested. For example, at a cell dose of $10^6$, median percentage engraftment was 13.3% (2.43%-49%) by IB injection compared with 1.2% (0.1%-4.6%) by IV delivery (p<0.01). The IB delivery method produced higher engraftment levels even when compared to double the number of cells infused IV. Engraftment percentage after IB injection of $5\times10^5$ cells was 4.28% (0.001%-17.9%) vs. 1.2% (0.1%-4.6%) after injection of $10\times10^5$ cells IV (Figure 4.1).

The superior engraftment by IB injection suggests there is a considerable wastage of circulating HSPC that simply do not arrive in the BM in a timely fashion, hence this strategy could rescue the defective engraftment of cytokine activated hCD34+ cells.

4.3.2 Expanded cell engraftment after IB infusion

The IB injection route was then used to deliver fresh or cultured hCD34+ cells (in a range of doses) into irradiated NOD/SCID mice. Results indicated that engraftment levels were notably lower for cells that had been exposed to cytokines for 72 hours, at all cell doses tested (Figure 4.2). At a cell dose of $10^5$/animal, median engraftment of cytokine activated cells was 0.73% (0.001%-10.93%), compared with 13.3% (2.43%-49%) for fresh cells (Mann-Whitney test, p<0.05).
Figure 4.1: Engraftment after IV or IB injection of fresh HSPC. Freshly isolated hCD34+ cells were transplanted, by IV or IB delivery, at the indicated cell doses, into sublethally irradiated NOD/SCID animals. Engraftment was assessed by FACS analysis of BM harvested at 8-10 weeks post–transplantation. Data are presented for individual animals. Broken grey lines demonstrate median IV engraftment and solid black lines median IB engraftment. Mann-Whitney test, p<0.01 for cell dose 10x10^5.
Figure 4.2: Engraftment of fresh and cytokine cultured hCD34+ cells following IB delivery at different cell doses. Data are presented for individual animals. Broken grey lines demonstrate median cultured cell engraftment and solid black lines median fresh cell engraftment. Mann-Whitney test, p<0.001 and <0.05 for cell doses 5x10^5 and 10x10^5 respectively.
Stem cell expansion with cytokines is often associated with dilution of the stem cell population. For this reason the expanded equivalent of the starting population is often used in these assays to correct for any dilution-based errors. Thus, in a separate series of experiments, twice the cell dose was used when cultured cells were injected. This cell dose was adequate to cover cell expansion, as the total cell number increased by a maximum of 1.6-fold over 72 hours in culture, in these experiments. Infusion of double the number of cytokine expanded cells (in comparison to the fresh cells), however, did not rescue the levels of engraftment (Figure 4.3). More specifically, after infusion of $5 \times 10^5$ fresh cells, the engraftment levels were 5.36% (0.001%-50%) compared to 0.001% (0.001%-1.17%) for the same cell dose after culture for 72 hours in cytokines ($p<0.001$). Even at a cell dose of $10^6$, engraftment of cultured cells was 0.73% (0.001%-10.93%, $p<0.05$, when compared to engraftment of $5 \times 10^5$ fresh CD34$^+$ cells) (Figure 4.3).

To study the kinetics of the loss of engraftment following exposure to cytokines hCD34$^+$ cells were cultured in the standard cytokine cocktail for 0, 24, 48 and 72 hours before IB transplantation. These experiments revealed that there is an 11-fold decrease in engraftment after 24 hours of cytokine activation, reaching its nadir (0.04%, $p<0.001$) at 72 hours (Figure 4.4).

**4.3.3 Engraftment at injection site and HSC transmigration**

The IB transplantation protocol has the additional advantage of providing information not only for the retention capacity of the infused cells but also for their migratory ability to other haemopoietic sites after engraftment. Using this model we investigated the engraftment levels of fresh and cultured cells at the site of injection (right tibia) which is a direct measurement of their retention in the BM. As seen in Figure 4.5, the engraftment defect persisted even when analysis was confined to the site of injection. Engraftment of activated cells in the injected bone was significantly lower than that of fresh
Figure 4.3: Reduction in IB engraftment of cytokine activated cells is not overcome by delivering higher cell doses. Irradiated animals were injected IB with $5 \times 10^5$ fresh and $5 \times 10^5$ or $10 \times 10^5$ cytokine activated hCD34+ cells. Engraftment was assessed at 8-10 weeks post injection by quantifying percentage of live cells obtained from flushed BM that were CD45⁺CD33⁻CD19⁻ or CD45⁺CD33⁺CD19⁺. Activated cells have significantly lower engraftment even when double the dose of fresh cells is injected. Mann-Whitney test, *p<0.05, ***p<0.001.
Figure 4.4: Kinetics of IB engraftment. HCD34⁺ cells were injected (IB) fresh (0 hours) or following culture in cytokines for 24, 48 or 72 hours at cell doses of 5 (●) or 10 × 10⁵ (○). Data are presented for individual animals with medians indicated for each timepoint. Mann-Whitney test, p<0.01 between 0 and 24 hours and between 0 and 48 hours and p<0.001 between 0 and 72 hours (for both doses analysed together). P<0.5 between 0-48 hours and 0-72 hours for the 5×10⁵ dose analysed separately.
Figure 4.5: Engraftment in injected bone. Engraftment of fresh and cytokine cultured hCD34+ cells was analysed in the injected right tibia only. 5 (●) and 10x10^5 (○) cell doses are shown. Data are presented for individual animals with medians indicated. P<0.05, Mann-Whitney test.
cells. Fresh cells had median engraftment of 14.4% (0.08%-71.2%) at the injected bone whereas cultured cells achieved 0% (0%-9.28%) median engraftment suggesting that these cells fail to be retained within the BM, even when directly delivered there.

We next compared the engraftment levels between the injected right tibia, and the other, non-injected bones for fresh and cultured HSC. In Figure 4.6 engraftment at the non-injected bones is expressed as a percentage of the engraftment levels of the injected bone for each animal. Transmigration to other bones is reduced for both fresh and cultured cells. This effect appears to be accentuated for the cytokine exposed cells although the difference (between fresh and cultured cells) is not statistically significant. Statistical significance could potentially be achieved if more animals were used in these experiments.

4.4 Discussion

IB delivery of HSC has a number of advantages over IV injection for the assessment of engraftment. It allows for the detection of distinct stem cell populations that would normally be lost during the homing process. It allows for analysis of HSC retention in the BM and the assessment of the transmigration capacity of the transplanted cells to other haemopoietic sites whilst increasing the overall engraftment levels in the recipients. Cytokine expanded MPB CD34+ cells have been shown in a number of reports (Van der Loo & Ploemacher, 1995; Szilvassy et al, 2001; Ahmed et al, 2004) including this work, to have reduced ability to localise and home to the haemopoietic niches (section 3.2.5). For this reason the IB infusion protocol was used to study the long-term engraftment of cytokine expanded cells in a system where the homing process is bypassed.
Figure 4.6: Transmigration of fresh and cultured cells. The engraftment levels in the injected tibia versus the non-injected bones, for fresh (top panel) and cytokine cultured (bottom panel) cells was assessed, at a cell dose of $5 \times 10^5$. Engraftment in the non-injected bones is expressed as percentage of the engraftment levels in the injected tibia of each animal, which is normalized to 100%. Data given for individual animals.
HSCT with IB cell infusion has, so far, only been used with different cell populations of CB samples. Therefore the initial aim of this work was to assess the robustness of this assay using MPB CD34+ cells, which is the most abundantly available cell source in clinic and the cell source used in this project. IB delivery increased the levels of engraftment of fresh MPB CD34+ cells, across a range of cell doses. At a cell dose of $10^6$ cells/animal there was an 11-fold increase at the levels of engraftment when the cells were delivered IB when compared to IV infusion (p<0.01, Mann-Whitney test). The IB delivery method produced higher engraftment levels even when compared to double the number of cells infused IV. Overall IB injection of MPB HSPC demonstrates similar advantages to what has been reported for CB HSPC in terms of long-term engraftment.

The next step was to test whether IB infusion of ex vivo expanded HSPC could improve their engraftment in comparison to the levels achieved by freshly isolated cells. Fresh and cultured cells were injected IB in sublethally irradiated NOD/SCID mice at several cell doses. We found that cytokine activated cells had consistently lower levels of engraftment compared to fresh cells at all cell doses tested. Furthermore, fresh cells engrafted significantly better even when half the cell dose was administered compared to cultured cells. These data demonstrate that expansion of MPB CD34+ cells with cytokines not only reduces their ability to home to the haemopoietic tissue of the recipient but also substantially reduces their ability to attach in the BM microenvironment and their retention there, which is essential for long-term engraftment. Of interest, this loss of attachment happens within 24 hours of exposure of the cells to the cytokine cocktail and is not related to cell expansion.

Successful engraftment after IB HSPC injection requires not only the retention and engraftment of the infused cells at the injected bone but also their
transmigration and colonisation of additional haemopoietic sites. This model allows for the analysis of these two processes separately.

To exclude the confounding effect of subsequent migration of transplanted cells into non-injected bones, we initially confined our engraftment analysis to the injected bones. When we analysed the injected bone alone we found a significant reduction at the levels of engraftment of cultured cells, in comparison to fresh cells. Yamamura and colleagues have reported no significant differences in CB AC133+ engraftment at the injected bone after 5-day culture in cytokines (Yamamura et al., 2008). These differences could be related to the cell population and/or conditions of activation or the specific cell population used for transplantation. In this work, the significant reduction in long-term engraftment of MPB CD34+ cells at the injection site further supports the hypothesis that cytokine activation induces an additional attachment defect to these cells.

The egress of engrafted HSC from haemopoietic tissue, as well as their subsequent circulation and colonisation of other haemopoietic tissues is a process separate to the initial lodgment and engraftment post infusion, and hence is likely to be governed by a different set of factors. As discussed in section 1.7.1, this process may serve to filter the cells that are permitted to re-enter haemopoietic tissue, so as to prevent de-differentiation of foreign or mutated cells, which could lead to malignancies. In this work, IB injected HSC failed to successfully translocate to other haemopoietic tissue, an effect that is accentuated after exposure to cytokines (Figure 4.6). Similar results for ex vivo expanded cells have been demonstrated in CB by Yamamura and colleagues (Yamamura et al., 2008). These observations could be related to a cytokine-induced downregulation of receptors that signal protection from immune clearing or niche filtering (the “don’t eat me” signals as they are referred to by the Weissman group), which prevents them from re-entering into the niches once they have been released into circulation in the circadian
fashion previously discussed. Further work in this aspect of cell engraftment could test for the expression of these receptors before and after exposure to cytokines and study whether induced expression in cultured cells can improve their transmigration to other haemopoietic tissue, utilising this IB transplantation model.

The main observation in this section is that exposure to cytokines causes a rapid reduction in the ability of MPB HSPC to be retained and subsequently engraft in the BM microenvironment even when directly delivered there. Although this points to a cytokine-induced attachment defect, the reasons behind it remain unclear and therefore the next chapter describes a novel ex vivo model designed to represent HSPC attachment in the BM.
5 Development of an *ex vivo* attachment model

5.1 Introduction

5.1.1 Two dimensional (2D) BM models

In the recent years, mimicry of the BM has been attempted through a variety of *in vitro* models mainly aiming to aid HSPC proliferation. Initial attempts involved the use of co-culture systems of HSPC with stromal cells (Dexter *et al*., 1977; Gartner & Kaplan, 1980). These efforts have been hampered by the fact that the supporting cells introduce foreign human or animal antigens to the product, which could render it inadequate for transplantation (reviewed in Verfaillie, 1994). Autologous feeder layers can be used instead, however, exposure of the patient to chemo or radiotherapy for myeloablation, prior to harvesting, disables the supportive function of these cells (Mayani, 1996) and in other cases the disease progression has rendered them ineffectual (Bhatia *et al*., 1995). Moreover, 2D cell co-cultures, with a fibroblastic feeder layer, for example, induce an artificial polarity between the lower and upper surfaces of these normally non-polar cells which alters their function, morphology and biology (Friedl & Bröcker, 2000). For these reasons, and given the 3-dimensionality (3D) of the BM *in vivo*, research soon focused on the development of 3D culture systems.

5.1.2 3D BM models

Culture of HSPC in 3D matrixes has seen much progress in the last 20 years with a great number of novel models developed (Naughton & Naughton, 1989; Naughton *et al*., 1990, 1991; Wang *et al*., 1995; Bagley *et al*., 1999; Tomimori *et al*., 2000; Wang *et al*., 2001; Banu *et al*., 2001; Xiong *et al*., 2002; Tun *et al*., 2002; Kim *et al*., 2003; Liu *et al*., 2009; Blanco *et al*., 2010; Mortera-Blanco *et al*., 2011; Leisten *et al*., 2012). All these artificially created 3D culture
platforms for haemopoietic cells have three basic components: the stem cells themselves, the scaffold supporting the stem cells, and the media which is necessary for their survival and proliferation. Additional components may include feeder cell layers (Wang et al., 1995; Tomimori et al., 2000; Sasaki et al., 2002; Xiong et al., 2002; Sasaki et al., 2003) or bioreactors (Wang et al., 1995; Tomimori et al., 2000; Jelinek et al., 2002; Mantalaris et al., 2004). In terms of the scaffolds used, researchers have opted for natural materials, such as cellulose and collagen (Wang et al., 1995; Tomimori et al., 2000; Jelinek et al., 2002; Mantalaris et al., 2004; Leisten et al., 2012) or synthetic materials, such as porous polyvinyl formal, tantalum coated porous biomaterial and polyester nonwoven fabric porous disc carriers (Li et al., 2001; Tun et al., 2002; Sasaki et al., 2002, 2003).

These 3D systems offer great advantages in comparison to their 2-dimensional equivalents. Their porous scaffolds allow for the culture of higher numbers of cells, compared to the conventional monolayer (Li et al., 2001). The combination of HSPC with feeder layers in a 3D structure more closely mimics the haemopoietic niche in vivo, which aids the multilineage expansion and self-renewal of the cells (Wang et al., 1995; Jelinek et al., 2002; Mortera-Blanco et al., 2011; Leisten et al., 2012). Additionally, bioreactors, where used, permit better control of the culture parameters and often have automated feeding systems which reduce contamination risks. Finally these systems allow for manipulation of their components such as the type of scaffold (Lutolf & Hubbell, 2005), feeder layer and culture medium which can subsequently alter the function and type of cells produced.

The aim in these systems is to maximise the expansion of transplantable HSPC. Therefore the focus has been placed on control, optimisation and standardisation for scale-ups, and where possible a “hands-off” operating system. Such characteristics would make the end-product predictable, reproducible, free of contaminants, and as such, suitable for therapeutic
applications to humans. For that reason much care has been taken to avoid the use of foreign antigens that would induce an immune reaction to the recipient. Neutral materials are used where possible and the inclusion of other cell types is minimised. While this strategy renders these systems potentially translatable to the clinic, it significantly reduces the accuracy of their representation of the haemopoietic niches \textit{in vivo}, which include a great number of extracellular matrix proteins and a variety of supportive cell types.

\subsection*{5.1.3 A new 3D model to study cellular interactions in the BM}

Engraftment of HSPC in the marrow microenvironment requires not only the trafficking and homing of the infused cells but also their lodgment and attachment at these sites. The attachment step, which is essential for long-term engraftment, is determined through complex, bidirectional interactions between the transplanted cells and the various cell components and extracellular matrix proteins in the niche. The study of these adhesive interactions, in order to better understand the factors affecting the engraftment of normal MPB, CB or BM HSPC, is a significant research target with potential translational prospects in clinical transplantation.

Cytokine activation of MPB CD34$^+$ cells leads to a reduction in long-term engraftment (Hao \textit{et al}, 1996; Guenechea \textit{et al}, 1999; Szilvassy \textit{et al}, 2000; Young \textit{et al}, 2001) which is partly related to a reduction in the levels of homing to the BM (Liu \textit{et al}, 2003; Ahmed \textit{et al}, 2004). The data discussed in Chapter 4 demonstrate that, even when the defective homing process is bypassed, and the cells are delivered directly in the BM cavities, they fail to engraft successfully, pointing to an additional cytokine-induced retention deficiency. Therefore, in order to study, manipulate and potentially improve the function of the cytokine expanded cells after transplantation, one needs to separately investigate their ability to attach in the BM microenvironment. For these reasons the next step was to produce an \textit{ex vivo} model which would
allow us to closely study the attachment of haemopoietic progenitors in the marrow.

Suggested cellular components of HSC niches include spindle-shaped osteoblasts (Zhang et al., 2003; Calvi et al., 2003), reticular cells (Sugiyama et al., 2006), osteoclasts (Kollet et al., 2006), nestin+ perivascular cells (Méndez-Ferrer et al., 2010) and a variety of ECM proteins (reviewed in Scadden, 2006). The complexity of the BM microenvironment both in terms of composition as well as localisation of cells and ligands is poorly represented in the models described above. In this work, where the aim was to study the attachment of HSPC within the BM, we chose to produce a novel model, which would more accurately represent these interactions as it utilises the long bones of rats. Thus, in this model, the basic components of the BM microenvironment should be present at the right positions and in the appropriate concentrations. Human HSPC are infused and incubated in the bones ex vivo. The adherent population is then harvested and progenitor attachment is quantified with colony assays.

5.1.4 Comparison of the ex vivo model with existing in vitro and in vivo assays

As a means of studying the retention of HSPC in the BM, this model fills a gap between the in vitro adhesion assays and the in vivo models of engraftment. In vitro adhesion assays allow for the analysis of the interaction of specific ligands with HSPC. Whilst they answer particular questions about the adhesive interactions of HSPC with niche ligands, these assays use immobilised recombinant proteins on culture plates at concentrations that are only assumed to be representative of those found in vivo. In addition to this, the incubation, wash and harvesting protocols do not necessarily correspond to similar processes in vivo. Moreover, only the already described ligands can be tested in these assays and any other contributing factors are excluded.
Therefore while adhesion assays are a very useful tool to dissect certain pathways, they are not representative of the attachment *in vivo*.

On the other hand, *in vivo* transplantation models are the most accurate measurement of HSPC engraftment but they cannot easily answer more specific questions. The long-term engraftment levels depend on a number of factors, in addition to cell attachment, such as homing, transmigration, survival, proliferation and differentiation ability of the transplanted cells. An *ex vivo* model that would directly investigate the attachment ability of infused HSPC could give specific information regarding the effect of different culture conditions or treatments on the retention of the progenitors, but in a natural, inclusive and representative environment. Additionally, this model minimises animal use in comparison to the standard *in vivo* models, as each bone is used separately to test progenitor attachment and therefore four data points are collected from each animal.

5.1.5 Choice of species and age

Much of the research in the biology and function of human HSPC has utilised the mouse xenotransplantation system. A number of immunodeficient mouse models have been produced to study and support the mobilisation, homing and engraftment of HSC (section 1.2.2.2). Subsequently the cross-reactivity of the human receptors with mouse ligands has been well described. The *ex vivo* assay described here utilises the long bones of rats for infusion, incubation and harvesting of HSPC and was chosen primarily for the difference in size, compared to mouse models.

In this assay, the long bones (femurs and tibiae) are removed from the animal and the ends are pierced carefully with a 26 gauge, short (12mm), needle. The resident marrow is flushed and human hCD34+ cells are infused and incubated. This process must have minimum impact on the bone as any cracks would affect the architecture of the bone and would also lead to cell
loss through leakages which could potentially underestimate attachment levels. The non-adherent cells are flushed gently with medium and the adherent cells are harvested with two vigorous washes. Due to the amount of manipulation and the technicalities of this protocol the mouse bones would be too small to safely carry out the assay. Furthermore the animals used in this protocol need to be around 3-weeks old, for reasons explained below, which would make the mouse bones very difficult to handle and manipulate due to their size and fragility. Additionally the conventional needles available would cause a considerable amount of damage when introduced to such small bones, due to their relatively large size. We therefore opted for the use of rats for these assays.

The age of the animals used was a factor which was optimised in the initial experiments. It was found that, after about 4 weeks of age, the bones of the animals become ossified to the extent that any punctures, at either end, would cause them to crack which would render them unusable. Up to the age of three weeks, however, the ends of the bones could be easily pierced while retaining a tight seal and preventing loss of the cell suspension infused. Moreover it has been shown in humans, that the haemopoietic marrow in young subjects is more uniformly distributed throughout the length of long bones (Moore & Dawson, 1990; Niu et al, 2007). That would ensure that the infused cells would come in contact with the components of the haemopoietic niches through the whole length of the bone.

Rat models have been used extensively for the investigation of human stem cell behaviour in a variety of conditions such as stroke (Smith et al, 2011), optic chiasm demyelination (Pouya et al, 2011), hepatic cirrhosis (Bassiouny et al, 2011), spinal cord contusion injury (Niapour et al, 2011) and cerebral ischemia (Horita et al, 2006). Moreover, MPB CD34+ cells have been transplanted in rats for tissue repair after myocardial infarction (Sondergaard et al, 2009). The Sprague Dawley (SD) rat in particular is a breed of albino rat.
that has been used extensively in medical research mainly for its calmness and ease of handling. This was the only rat species bred by the UCL Biological Services. Any other type would have to be ordered in at a minimum age of 3 weeks and allowed to settle for 5-6 days before they could be used, which would make them too old to use in these assays, for reasons discussed above. Furthermore SD rats have been regularly used to test the engraftment of BM progenitors (MSC) for tissue repair purposes (Spees et al, 2007; Zhao et al, 2008; Lee et al, 2010b; Tong et al, 2011). In xenotransplantation experiments SD rats have been used to test the engraftment of human MSC (Muñoz-Elias et al, 2004) and CB CD34+ cells (Pan et al, 2005) in the brain. Finally, a great degree of sequence homology is observed for some of the most well characterised proteins in HSC engraftment in the haemopoietic niches, between human and (SD) rat (Table 5.1). Based on these considerations the long bones of young SD rats were utilised for the development of an \textit{ex vivo} model of the BM.

\section*{5.2 Protocol}

\subsection*{5.2.1 Bone preparation}

Male, 3-week old SD rats were irradiated (6Gy) 24 hours prior to sacrifice (by CO$_2$ inhalation). Only animals weighing between 50-100g were used. The femurs and tibiae were excised in a sterile environment at the animal facility as described in the General Methods section 2.3.2 and were placed in tubes containing 5ml RPMI/10\% FCS with 20U/ml heparin (Transfer medium, Appendix I).

The bones were quickly transferred to the Cancer Institute and were placed in Transfer medium in a 6-well plate until they were further processed. Any surrounding tissue was removed taking care to maintain the bones intact. Using a 26 gauge, 12mm needle the bones were pierced on one end until the needle
Table 5.1: Percentage protein homology between human and Sprague-Dawley (SD) rat.
The sequences of proteins reported to be significant in the interaction of HSC with the haematopoietic niche were compared using Blast® between Homo sapiens and Rattus norvegicus (SD).

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>% HOMOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-cadherin</td>
<td>97</td>
</tr>
<tr>
<td>SDF-1a</td>
<td>92</td>
</tr>
<tr>
<td>CXCR4</td>
<td>91</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>77</td>
</tr>
<tr>
<td>VLA4 (ITGA4)</td>
<td>84</td>
</tr>
<tr>
<td>VLA5 (ITGA5)</td>
<td>90</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>60</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>86</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>70</td>
</tr>
<tr>
<td>CD44</td>
<td>89</td>
</tr>
<tr>
<td>ICAM1</td>
<td>51</td>
</tr>
<tr>
<td>LFA1 (ITGB2)</td>
<td>81</td>
</tr>
<tr>
<td>ANG1</td>
<td>97</td>
</tr>
<tr>
<td>TIE2</td>
<td>93</td>
</tr>
<tr>
<td>PTH</td>
<td>71</td>
</tr>
</tbody>
</table>
reached the cavity. The needle was then removed and replaced and another incision was made at the other end of the bone. The resident marrow was then flushed using 5ml HBSS. The incision sites were kept constant between bones and animals. At the top of the femur the needle was inserted between the head and the greater trochanter. At the lower end of the femur the needle was inserted at the intercondylar fossa between the medial and the lateral condyle. After the fibula was removed, the top of the tibia was pierced at the intercondylar eminence. At the bottom, the tibia was punctured at the centre of the inferior articular surface. The positions are indicated at Figure 5.1. The needles were kept in place for the next step.

5.2.2 Cell infusion

Once the resident marrow was flushed and the bone cavity was clear (as determined by visual inspection), a 5ml and a 1ml syringe were placed on the needles at either end of the bone. One ml of HBSS was then passed from the 1ml syringe through the bone and into the 5ml syringe in a “push-pull” fashion. This process ensured that the needles were not blocked and that there were no cracks or leakages in the bone. If necessary any cracks were sealed with bone wax (Ethicon, Aunea).

The bones were then placed in large cell culture dishes, divided according to the conditions to be tested. Where possible the 4 bones from each animal would be divided between the different conditions to minimise the effect of biological variability on the data.

The cell suspensions were prepared in IMDM/2% FCS, at a concentration of $5 \times 10^5$/ml (unless otherwise stated). The cells were infused using two 1ml syringes. The two syringes were placed at either end of the bone and the cell suspension was pushed from one of the syringes through the bone and into the empty syringe on the other side (Figure 5.2). The infusion was stopped
Figure 5.1: Injection sites in the rat model. The arrows indicate the sites where the needles are inserted on both ends of the femur (left panel) and the tibia (right panel).
Figure 5.2: HCD34* cell infusion in the bones. The rat femurs and tibias were excised, tissue removed and resident marrow flushed out. The ends have been punctured with 26 gauge needles and HBSS medium was passed through the bones to check the flow and water-tightness of the system. Finally the cells were infused from an 1ml syringe through the bone and into another syringe at the other end of the bone, as shown in the picture.
when the cell suspension appeared in the empty syringe. That process ascertained that the whole of the BM cavity had been filled with the cell suspension. The volume of the cavities, as estimated through this process, is approximately 80µl. Finally the syringes were removed and the bones, with the needles still in place, were incubated at 37˚C for 90 minutes (Figure 5.3). Medium was added around the bones to keep them moist during incubation.

5.2.3 Harvesting and assessment of progenitor attachment

After incubation the looser of the two needles was removed and a 5ml syringe containing 3.5ml of HBSS was placed on the remaining needle. Care was taken to include approximately 1ml of air in the syringe. This process ensures that no medium is left in the dead space of the needle or inside the cavities after flushing. The first 2ml was slowly passed through the bone and into a sterile collection tube, to wash off the non-adherent cells. The remaining 1.5ml was vigorously pushed through to harvest the adherent cells which were collected in a separate tube. Another 2ml of HBSS was added to the syringe (plus 1ml of air) and the hard wash was repeated to collect any remaining attached cells. Thus in total, the non-adherent cells were collected in 2ml volumes whereas the adherent cells in 3.5ml of HBSS.

In order to assess the levels of progenitor attachment a proportion (300µl) of the attached cell suspension was seeded in human specific methylcellulose (supplemented with GM-CSF, SCF and IL-3). We performed optimisation experiments to establish the appropriate cell volume to seed and whether the non-adherent population could also be used to measure attachment. We found that the non-adherent cell suspension showed high variability in the number of colonies produced due to the fact that some of the starting cell suspension had remained in the dead space of the needles and was washed off into this suspension, significantly altering the cell numbers. For this reason attachment was measured from the adherent cell suspension only,
Figure 5.3: Femurs and tibias prepared for incubation with infused hCD34+ cells. After the cells were infused into the bones the syringes were removed. Medium was added around the bones to keep them moist during incubation. The needles were kept in place and the bones were incubated for 90 minutes at 37°C.
which had no such complications, and for which the values acquired were more consistent between bones and experiments.

The colonies produced were scored 14 days later. The numbers were normalised to a CFC output of 120 per 2,000 CD34\(^+\) cells to control for variability in donor clonogenicity. As the size of the cavities differs depending on the bone size we also normalised the values acquired to an average animal weight of 80g, assuming the animal weight will be proportional to their bone size. The results are expressed as number of adherent CFU-GM/bone and are presented separately for each bone.

**5.3 Results**

**5.3.1 Animal irradiation**

Initial optimisation experiments focused on the need for irradiation prior to cell infusion. Animals were exposed to an irradiation dose of 6Gy, a dose that has been tested in rats in a variety of disease models (Ramadan et al, 2001; Demirel et al, 2009; Vinchon-Petit et al, 2010; Kilciksiz et al, 2011). In order to test the effects of irradiation we compared the attached progenitor levels between animals that had been irradiated or not, after they were infused with a hCD34\(^+\) cell suspension or medium only. As shown in Figure 5.4, in the absence of irradiation, the levels of recovered CFC are much higher in bones infused with hCD34\(^+\) cells (median 1725, range 1418-2398 for non-irradiated animals vs. 645.8, range 292.3-1124 for irradiated animals). Moreover, when no cells were infused, no CFC were recovered from the irradiated animals, whilst colony growth was observed for the non-irradiated animals (Figure 5.4). This suggests that the resident rodent progenitors, which form a great proportion of the adherent cell suspension, are capable of producing colonies in the “human specific” methylcellulose, skewing the results. Therefore, pre-irradiation of the animals is necessary for the
Figure 5.4: Irradiation effect on the ex vivo attachment model. The numbers of attached CFC are shown for animals that were not irradiated (○) or were irradiated with 6Gy (▲) prior to cell infusion. Data shown for each bone with medians indicated. **p<0.01, Mann-Whitney test.
specificity of this model. Total body irradiation prior to transplantation is used in the majority of the well-established human to mouse xenotransplantation models (Verstegen et al, 1998; Kollet et al, 2001; Uchida et al, 1994). Myeloablation prior to cell infusion is also the standard protocol in HSPC transplantation in the clinic.

5.3.2 Dose response

Next, the optimal concentration of cells to be infused in the bones was established by performing dose response experiments. Zero, 0.5 and 1x10^6 cells/ml suspensions of fresh hCD34^+ cells were prepared and injected into the bones as previously described (section 5.2.2). As shown in Figure 5.5 the model is responsive to the increasing cell doses. The bones injected with medium alone had no attached progenitors whereas the bones injected with the 0.5x10^6/ml cell dose had 403.2 (70.04-974.3) and the bones infused with 1x10^6/ml had 862 (292.3-1124) attached progenitors. Due to the smaller deviation of the values acquired for the 0.5x10^6/ml cell dose this dose was selected for all future experiments, so as to maintain consistency between results.

5.3.3 Effect of cytokine activation on HSPC attachment

Following optimisation of the protocol, the model was used to test whether cytokine activation impairs the attachment of progenitors in the BM. Fresh and 72 hours cultured CD34^+ cells were infused in the bones and progenitor adhesion was measured as previously described (5.2.3). As shown in Figure 5.6, culture in cytokines reduces the attachment of haemopoietic progenitors in the BM (385.4, range 48.62-1124 for fresh cells vs. 182.1, range 23.05-384.3 for cultured cells). The attachment of fresh and cultured CFC was also compared across a range of cell doses, to determine whether the differences observed were retained irrespective of the cell dose. As shown in Figure 5.7 the difference between fresh and cultured progenitors, in terms of their
Figure 5.5: Dose response in the ex vivo attachment model. Cell suspensions of 0, 0.5 and 1x10⁶ hCD34⁺ were infused in the bones and the levels of progenitor attachment were measured. Values for individual bones are shown with medians indicated. ***p<0.001, Mann-Whitney test.
Figure 5.6: Fresh and cultured cell attachment in the ex vivo model. Fresh or cytokine cultured (72 hours) hCD34+ cells were infused in the bones at a cell dose of 0.5x10⁶/ml. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. ***p<0.001, Mann-Whitney test.
Figure 5.7: Dose response in attachment of fresh and cultured CFC. Fresh or cytokine cultured (72 hours) hCD34+ cells were infused in the bones at cell doses of 0, 0.5 and 1x10^6/ml. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. *p<0.05, **p<0.01, ***p<0.001, statistical analysis by Mann-Whitney test.
attachment in the BM, is retained for the 0.5 and 1x10^6/ml cell doses. These observations directly confirm the hypothesis made from the intrabone engraftment results described in Chapter 4, i.e. that culture in cytokines impairs the attachment of haemopoietic CFC in the BM microenvironment.

The attachment defect described above is evident after culture of hCD34^+ cells in cytokines for 72 hours. The next step was to describe the kinetics of this phenomenon. HCD34^+ cells were cultured in the standard cytokine cocktail for 0, 16, 24, 48 and 72 hours. The cells were then infused in the bones and progenitor adhesion was measured. As shown in Figure 5.8, there was a 60% decrease in the numbers of attached progenitors within 16 hours of exposure of the cells to the cytokines (856.7, range 645.8-1022, for fresh cells vs. 348.2, range 43.75-514.1 after 16 hours of culture), with a nadir reached after 48 hours (158.9, range 51.21-268.8).

5.3.4 CXCR4 blockade

The significance of the CXCR4/SDF-1 interaction in the adhesion and retention of haemopoietic progenitors has been described in many experimental contexts by various groups (reviewed in Sharma et al, 2011). The in vivo relevance of this model was explored by testing the effect of CXCR4 blockade. As shown in Figure 5.9, CXCR4 blockade significantly reduces the attachment of both fresh and cultured progenitors. The number of attached CFC for fresh cells was 448.4 (189.4-1124) but was reduced to 259 (161-608) after CXCR4 blockade. In cultured progenitors, CXCR4 blockade reduced the attached CFC numbers from 264.6 (186-383.7) to 123.6 (36-275).

Confirmation that the reduction observed was receptor specific and not treatment related was obtained by using an isotype control antibody. As shown in Figure 5.10 only the specific blocking antibody (and not the isotype control) has an effect on the attachment of progenitors in the BM in comparison to the untreated cells. These experiments underline the
Figure 5.8: Timecourse in attachment of fresh and cultured CFC. HCD34+ cells were cultured for 0, 16, 24, 48 and 72 hours in cytokines and were then infused in the bones at a cell dose of $0.5 \times 10^6$/ml. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. *$p<0.001$, Mann-Whitney test.
Figure 5.9: Effect of CXCR4 blockade on CFC attachment. Fresh or cytokine cultured hCD34+ cells were pre-incubated with anti-CXCR4 antibody (15µg/ml) or PBS, as indicated, for 1 hour on ice, before incubation in the bones. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. *p<0.05, **p<0.01, statistical analysis by Mann-Whitney test.
Figure 5.10: Anti-CXCR4 or isotype control antibody treatment. Fresh hCD34⁺ cells were pre-incubated with anti-CXCR4 antibody (15µg/ml), the isotype control (15µg/ml), or PBS as indicated, for 1 hour on ice, before incubation in the bones. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. **P<0.01, Mann-Whitney test.
importance of the CXCR4 receptor in the attachment of HSPC in haemopoietic tissue. Furthermore, loss of attachment with CXCR4 blockade, in this context, supports the specificity and biological relevance of this model.

5.4 Discussion

This chapter describes the development of a novel ex vivo model that utilises the long bones of young rats. This is the first rat model that has been developed for the assessment of haemopoietic progenitor attachment in the BM. The model organism was selected mainly for practical reasons related to their age and subsequent size, as discussed in section 5.1.5. Although not as widely used as the mouse immunodeficiency models, rat models have been previously used to study the behaviour of human stem cells in general (Horita et al, 2006; Smith et al, 2011; Pouya et al, 2011; Bassiouny et al, 2011; Niapour et al, 2011) and haemopoietic stem and progenitor cells in particular (Muñoz-Elias et al, 2004; Pan et al, 2005; Spees et al, 2007; Zhao et al, 2008; Tong et al, 2011; Lee et al, 2010b; Tong et al, 2011).

Irradiation of the animals prior to sacrifice is essential for the specificity of this model. When the animals are not irradiated a variable number of resident rat progenitors survive the flushing process and produce colonies thus affecting the end results of the assay. Exposure to irradiation is expected to disrupt the architecture of the BM of the animals. However, ionising irradiation will also stimulate osteoblast proliferation within the BM and increase the SDF-1 levels, especially in the metaphysis, where osteoblast proliferation is most evident (Dominici et al, 2009). This will enhance CXCR4-dependent homing and repopulation of human HSPC into immunodeficient animals (Ponomaryov et al, 2000). These changes will undoubtedly affect the interaction of the infused cells with the BM surface. Irradiation, however, is the standard conditioning for the well tried and tested mouse xeno-transplantation models and the upregulation of SDF-1, in particular, is expected to attract and retain higher
numbers of circulating progenitors within the BM niches. Finally, myeloablation, often with irradiation, is a commonly used protocol prior to stem cell transplantation in the clinic. Therefore, conditioning of the rats with irradiation prior to the attachment assays renders the model more clinically relevant.

Flushing of the resident marrow is an essential step in this protocol as the BM cavity needs to be empty before the human HSPC are infused. This process is expected to damage, to some degree, the architecture of the BM microenvironment and remove some of the cells that would normally support the retention and engraftment of HSC \textit{in vivo}, such as stromal cells. Efforts were taken to minimise this effect by retaining the external integrity of the bone during processing and using short needles to reduce any damage to the internal architecture of the bones. Despite the expected loss of some of the vascular elements of the haemopoietic tissue, we presume that the bone surface remains, which would include osteoblasts and extracellular matrix proteins. The interaction of HSPC with these remaining elements in a natural, three-dimensional environment is still particularly informative in terms of their adhesive behaviour \textit{in vivo}.

This model was used to test and compare the attachment of fresh and cytokine activated progenitors. Culture of hCD34$^+$ cells in cytokines for 72 hours significantly reduced the levels of progenitor attachment in the BM (Figure 5.6). This drop in attachment happened within 16 hours of exposure of the cells to the cytokines and before expansion (Figure 5.8). Moreover, the possibility that these observations are dependent on cell differentiation is unlikely because the attached cell population is functionally defined with clonogenic assays.

The significance of the CXCR4/SDF-1 interaction in HSPC homing, engraftment and maintenance has been underlined by various studies (Kollet
et al, 2001; Lapidot & Kollet, 2002; Kahn et al, 2004; Sugiyama et al, 2006; Dar et al, 2006; Sugiyama et al, 2006; Jung et al, 2006; Sharma et al, 2011). In agreement to the published literature, in this model we found CXCR4 blockade to half the attachment of fresh MPB progenitors. Cytokine activation has been proposed to increase the relative importance of the SDF-1/CXCR4 interaction in homing for BM HSC (Bonig et al, 2006). Here we observed that blockade of CXCR4 in cytokine stimulated hCD34+ cells was able to further decrease the already reduced attachment, in comparison to fresh cells. This indicated that the CXCR4/SDF-1 axis is still active and essential for the attachment of HSPC in the BM, even after their exposure to cytokines.

In summary, a new ex vivo model was developed to represent progenitor attachment in the BM after transplantation. This model is more representative than the in vitro adhesion assays whilst it minimises animal use, as every bone can be used for a separate assay/condition. The protocol of this assay has been optimised in terms of pre-irradiation, bone processing, cell numbers infused and flushing techniques. Moreover the model has been shown to be responsive to CXCR4 blockade confirming its biological relevance. Imaging of the bone with the infused hCD34+ cells could provide additional information in terms of the specific sites where HSC are retained within the BM. Additionally this model can be used to establish the relevant significance of other pathways in cell attachment, by pre-incubation of the infused cells with specific blocking antibodies. It is also an ideal platform on which to test how different HSPC expansion protocols can impact on the resulting attachment, prior to testing these conditions in in vivo transplantation models. Similarly, pre-treatment of the animals with niche-modulating agents can be used to test for their effect on HSPC attachment.
6 Adhesion of HSPC to BM ligands

6.1 Introduction

6.1.1 Cytokine-induced changes in HSPC adhesion receptors

The effect of cytokines on the expression and function of adhesion receptors on haemopoietic cells has been investigated by several groups in the last 20 years (Salmi & Jalkanen, 1992; Lévesque et al, 1995; Kovach et al, 1995; Miyazawa et al, 1995; Gotoh et al, 1997; Becker et al, 1999; Peled et al, 1999a; Giet et al, 2001; Berrios et al, 2001; Kollet et al, 2001; Huygen et al, 2002; Yong et al, 2002; Gothot et al, 2003; Denning-Kendall et al, 2003; Foguenne et al, 2009). In these studies a variety of cytokine combinations, incubation times and cell sources have been used and subsequently the observations vary significantly.

6.1.1.1 Murine cells

Using the murine cell line, FDC-P1 the Quesenberry group observed fluctuating expression of VLA-4 after cytokine exposure, declining during the G\textsubscript{i}/S phase transition and rising again during S phase (Becker et al, 1999). In primary murine Lin\textsuperscript−/Sca-1\textsuperscript+ cells, the same group showed that culture with IL-6, IL-3, IL-11 and SCF for 48 hours decreased adhesion to human and murine fibronectin and collagen, while adhesion to the TC-1 stromal cell line and Dexter stromal cells was completely abolished after this incubation (Becker et al, 1999). Similar results were observed in Lin\textsuperscript−Ho\textsuperscriptb/Rho\textsuperscriptb murine cells whereby stimulation with the same cytokine cocktail reduced adhesion to fibronectin and laminin but increased adhesion to collagen I (Berrios et al, 2001). These changes in adhesive properties are correlated with a decrease in VLA-4 and VLA-5 expression, whereas other receptors such as CD44 and \(\alpha\_L\) show increased expression (Becker et al, 1999; Berrios et al, 2001).
6.1.1.2 Human cell lines

A similar decrease in $\alpha_4$ expression was observed in the human haemopoietic cell line HL-60 by the same group (Becker et al, 1999). A shorter (2 hour) exposure of the human haemopoietic cell lines MO7e and TF1 to IL-3, GM-CSF and KIT had no effect on either VLA-4 or VLA-5 expression (Lévesque et al, 1995). These cell lines demonstrate a transient increase in adhesion to fibronectin, which is maximal after 30 minutes and returns to basal levels after 2 hours of culture in cytokines (Lévesque et al, 1995). Adhesion of MO7e cells to fibronectin has also been shown to be VLA-4 and VLA-5 dependent and to increase after exposure to thrombopoietin or erythropoietin (Gotoh et al, 1997). Additionally, after exposure to SCF alone, MO7e cells have been reported to activate c-Kit mediated adhesion (Miyazawa et al, 1995) and to transiently increase their adhesion to HUVEC and VCAM-1-transfected Chinese hamster ovary (CHO) cells, followed by a decrease in adhesion that reaches its nadir by 24 hours (Kovach et al, 1995).

6.1.1.3 Human primary cells

Less data exist on the effects of cytokine exposure on adhesion in primary human haemopoietic cells. CB CD34$^+$ cells have been reported to increase $\alpha_4$ and $\alpha_5$ expression after exposure to SCF, TPO, Flt3L and G-CSF (Huygen et al, 2002; Foguenne et al, 2009). Similar results for VLA-4, VLA-5, L-selectin, LFA-1 and CD44 expression on CB HSPC after culture in SCF, TPO, Flt3L, and G-CSF have also been reported (Reems et al, 1997; Denning-Kendall et al, 2003). Huygen et al also observed that cycling CB progenitors transiently increase their adhesion to fibronectin whereas VCAM-1 adhesion is decreased (Huygen et al, 2002). Transmigration through both fibronectin and VCAM-1 coated membranes is reported to irreversibly decrease for cycling CB cells (Huygen et al, 2002). Using binding assays, that measure the attachment of CD34$^+$ cells to soluble VCAM-1, Foguenne et al show that
while fresh CB cells have low affinity for VCAM-1, culture with SCF, TPO, Flt3L, IL-6 and G-CSF, significantly increases their adhesion to VCAM-1. They attribute this effect to a supraphysiological increase in VLA-4 affinity, which also is responsible for a reduction in migration levels through VCAM-1 coated membranes (Foguenne et al, 2009). In uncultured human steady state BM CD34+ cells, that are sorted according to their cycle status, cycling cells were shown to express more VLA-4 and to adhere more to stromal cells compared to cells in G0/G1 (Yamaguchi et al, 1998). In agreement with this observation, previous work in our laboratory has demonstrated greater adhesion of unstimulated, cycling MPB CD34+ cells to endothelium compared to their quiescent counterparts. This was accompanied by higher VLA-4 and CXCR4 expression for cells in the S and G2/M stages of the cell cycle, whereas no difference was observed in other receptors such as CD31 and L-selectin (Yong et al, 2002). Cycling MPB CD34+ cells have also been shown to have increased binding to fibronectin, whereas VCAM-1 adhesion was unaffected after 48 hours in culture (Giet et al, 2001). CXCR4 expression was reported, by one group, to decrease after culture with Flt3L, SCF, TPO and IL-3 (Brenner et al, 2004). In contrast, we have observed upregulation of the CXCR4 receptor in MPB CD34+ cells after culture in cytokines (section 3.2.3 and Yong et al, 2002). Additionally, CXCR4 upregulation after exposure to cytokines (SCF and IL-6 or SCF alone) has been reported for human CB and BM CD34+ cells, and was associated with increased migration and engraftment (Peled et al, 1999b; Kollet et al, 2001).

The majority of these studies assay total number of cells but fail to determine the clonogenic potential of these cells. Moreover the adhesive properties of haemopoietic progenitors have been repeatedly shown to be highly dependent on the cell source. When comparing the behaviour of murine and human cells one has to take into consideration the important physiological differences between humans and rodents. For example, it has been shown...
that in mice the HSC compartment is divided into the “dormant” (5-10%) and the “activated” (90-95%) populations, the first of which enters cell cycle 1 every 21 weeks (Wilson et al, 2008). In humans this frequency is 1 in 40 weeks (Catlin et al, 2011). Such differences could explain the disparate results reported between murine and human progenitor behaviour after cytokine stimulation.

Focusing on human cells, either primary (Yamaguchi et al, 1998; Giet et al, 2001; Yong et al, 2002; Gothot et al, 2003; Foguenne et al, 2009) or cell lines (Kovach et al, 1995), cycling cells appear to be more adhesive to stromal cells and vascular niche components, although this effect appears to be transient (Kovach et al, 1995; Huygen et al, 2002). These observations do not explain the cytokine-induced loss of engraftment that has been observed after exposure of CD34+ cells to cytokines. Furthermore, only the Gothot group has partially investigated this phenomenon on MPB cells, specifically.

Moreover, all the published studies that have investigated HSPC adhesion in the context of cytokine activation have focused on stromal cells and ligands (such as Dexter cells, collagen and fibronectin). While these elements are important in the tethering of HSPC on the endothelium and homing to the BM, the same ligands may not mediate attachment and long-term retention of these cells within the haemopoietic BM niches. With the exception of VCAM-1, which has been described to be a component of vascular niches, to date other niche-specific ligands have not been examined for their importance in HSPC adhesion.

6.1.2 Chapter aims

We have shown that the loss of engraftment of MPB HSPC after cytokine stimulation is partly related to a loss of attachment of these cells within the BM cavities (Chapter 5). In the context of cytokine activation, there are important pathways that have not been examined until now, despite their
important roles in the haemopoietic niches, such as N-cadherin and osteopontin (section 1.6). The aim of this chapter was to study the cytokine-induced changes in specific adhesion to these and the more studied ligands (fibronectin and VCAM-1, section 1.6) specifically for MPB HSPC.

6.2 Results

6.2.1 Adhesion assay optimisation

The interaction of HSPC with putative niche components was investigated using static adhesion assays, on immobilised ligands, in vitro. Initial experiments focused on optimising the adhesion assay. Rather than directly measuring absolute number of hCD34$^+$ cells, we opted for clonogenic assays as readout, because this functional assay reflects the behaviour of the progenitors. Additionally, for every condition tested the specific adhesion was measured, which was defined as the percentage progenitor adhesion to each ligand minus the adhesion to control (PBS) coated wells. Preliminary experiments indicated that freshly isolated HSPC adhered to immobilised N-cadherin significantly more than to control (PBS). This substrate, therefore, was used to optimise all other parameters of the assay. The number of cells seeded per well, incubation time and wash protocol were optimised. The ideal coating concentration for each ligand tested was also established in separate experiments.

The plates used for the culture of cells in vitro are normally treated with “plasma discharge” which renders the normally hydrophobic polystyrene surface, hydrophilic. This treatment aids cell attachment and spreading, which improves cultures, but can affect the results of adhesion assays in which ligand-specific adhesion must be measured. Comparison of tissue culture (TC) treated plates (SPL life sciences) with non-TC treated plates (Nunc) revealed that background adhesion was significantly higher in the former (Figure 6.1). Background (PBS) adhesion to TC plates was 53.67 ± 0.3333%
Figure 6.1: Adhesion on TC-treated and untreated plates. Adhesion of fresh hCD34+ to PBS or N-cadherin was tested in assays performed on TC treated or untreated plates, as indicated. Specific adhesion is calculated by subtracting the background progenitor adhesion (PBS) from the adhesion to the ligand. Background adhesion is lower in the untreated plates thus increasing specific adhesion to N-cadherin. Mean ± SEM shown, n=3.
(mean ± SEM), which was significantly higher compared to non-TC plates where background adhesion was 4.688 ± 3.754% (p<0.01, paired Student’s t-test). As a result, specific adhesion to N-cadherin was underestimated when TC-plates were used (21% for TC plates vs. 39.582% for non-TC plates). For this reason non-TC plates were used in all subsequent adhesion assays.

Next, the medium in which the adhesion assay is performed was optimised by comparing two different media in which hCD34+ cells can be maintained, Iscove’s modified Dulbecco’s medium (IMDM) and X-VIVO 10. Specific adhesion was found to be higher when the assay was performed in IMDM (39 ± 1.5% compared to 21 ± 5.5% in X-VIVO 10) as shown in Figure 6.2.

The serum content of the medium in which the assay is carried out was also predicted to have an impact on the resulting adhesion and for that reason several FCS concentrations were tested for their impact on these assays. As seen in Figure 6.3 the use of 2% FCS results in higher specific adhesion in comparison to 0 or 10% FSC (46% vs. 24.11% or 22.44% for 0% or 10% FCS, respectively, p<0.05 for both).

Finally the volume of the cell suspension seeded in methylcellulose has an important impact on the assay readout as the colonies do not grow well if too many or too few cells are seeded. For this reason experiments were performed where different volumes were seeded in methylcellulose from the same cell suspension (Figure 6.4). The difference between control and test adhesion was statistically significant for all seeding volumes but the highest significance (p<0.001, Student’s t-test) and biggest effect size was observed when 20µl was seeded (31.54% specific adhesion vs. 14.94% or 9.65% for 10µl and 30µl, respectively).
Figure 6.2: Medium optimisation for adhesion assays. Adhesion of fresh HSPC to N-cadherin was assayed in X-VIVO or IMDM (2% FCS). Specific adhesion was calculated as shown in Figure 6.1. P<0.5, paired Student's t-test, mean ± SEM indicated, n=3.
Figure 6.3: FCS concentration in the adhesion assays. Specific adhesion of fresh HSPC to N-cadherin was assessed in the presence of 0, 2 or 10% FSC in IMDM. Mean ± SEM is shown, n=3. *p<0.05, Student's t-test.
Figure 6.4: Seeding volume titration for the adhesion assays. Fresh hCD34\(^+\) were allowed to adhere to N-cadherin or PBS. From the non-adherent cell suspension increasing volumes were seeded in methylcellulose as indicated. Specific adhesion was highest when 20\(\mu\)l of the non-adherent cell population was seeded in methylcellulose for CFC quantification. Mean ± SEM, n=3.
Therefore, the optimal conditions in which specific adhesion can be tested were established as: 70,000 cells per well in 150µl IMDM with 2% FSC, in 48-well non-TC treated plates, for 90 minutes at 37˚C. Three washes were performed to collect the non-adherent cells (300µl each) and 20µl of the non-adherent cell suspension was seeded in methylcellulose for colony quantification.

6.2.2 HSPC adhesion to BM ligands and the effect of cytokine culture

Based on the literature review summarised in section 1.6 the following ligands were selected to test the effect of cytokine culture on HSPC adhesion: N-cadherin, osteopontin, VCAM-1 and fibronectin.

As shown in Figure 6.5 freshly isolated progenitors have significant levels of specific adhesion to these four ligands. Adhesion of fresh progenitors to N-cadherin was 31.76 ± 1.993% (mean ± SEM) over background. Specific adhesion to osteopontin was 23.51 ± 1.677%, to VCAM-1 36.26 ± 2.432% and to fibronectin 25.03 ± 3.035%. The specificity of adhesion was further confirmed with receptor blockade experiments, discussed below (Figure 6.7).

After culture in cytokines for 72 hours HSPC adhesion to three of these four ligands significantly declined. More specifically, N-cadherin adhesion decreased to 19.70 ± 3.198% (p<0.01, Student's t-test) after culture. Similarly, osteopontin adhesion was reduced to 12.11 ± 3.526% (p<0.01) and VCAM-1 to 26.48 ± 2.658% (p<0.05). Adhesion of cultured progenitors to fibronectin was unaffected by cytokine culture (24.18 ± 6.309%, NS).

This reduction in the adhesion to N-cadherin, osteopontin and VCAM-1 reported in Figure 6.5 was observed after 72 hours of culture in cytokines. Timecourse assays demonstrated that this effect on progenitor adhesion becomes apparent from as early as 16 hours after exposure to cytokines
Figure 6.5: Adhesion of freshly isolated and cultured hCD34⁺ progenitors. Specific adhesion of fresh and cytokine cultured hCD34⁺ progenitors to N-cadherin, osteopontin (OPN), VCAM-1 and fibronectin. Each donor was tested in triplicate, n=10 for N-cadherin, n=5 for osteopontin and VCAM-1 and n=3 for fibronectin. Mean ± SEM is shown, *p<0.05, **p<0.01.
Adhesion to N-cadherin was reduced by 83% in the first 16 hours (p<0.0001, n=3, Student’s t-test), and similarly it was decreased by 90% for osteopontin (p<0.0005, n=3, Student’s t-test). A lesser effect was seen on progenitor adhesion to VCAM-1 which was decreased by 52% after 16 hours in cytokine culture (p<0.0001, n=3, Student’s t-test).

The specificity of adhesion to each substrate was confirmed with receptor-specific blocking strategies. Specific blocking antibodies against CD44 (2.5µg/ml) and VLA-4 (CD49d) (5µg/ml) were used to confirm the specificity of adhesion to osteopontin and VCAM-1 respectively. The hCD34+ cells (either fresh or cultured in cytokines) were incubated with the specific blocking antibodies for 40 minutes on ice prior to the adhesion assay. For N-cadherin a blocking peptide was used (Ac-CHAVC-OH) which has been reported to be a potent inhibitor of N-cadherin function (Williams et al, 2000). This is a cyclic peptide that forms a disulphide bridge between Cys1 and Cys5, blocking N-cadherin function after 15 minutes of incubation (1mM at 37°C). Fresh and cultured progenitor adhesion was measured with and without the blockade treatments on all three ligands. As can be seen in Figure 6.7 the blocking strategies significantly decreased the adhesion of fresh and cultured cells to the ligands tested (p<0.05 for all treated/untreated combinations, paired Students t-test). To demonstrate that the reduction observed with the blockade was specific to the pathway and not a treatment-induced effect, parallel adhesion assays were performed using isotype controls for the anti-CD44 (rat IgG2b) and anti–VLA4 (mouse IgG1) antibodies and a scrambled peptide for N-cadherin (Ac-CHGVC-OH). These treatments had no effect on the specific adhesion of fresh progenitors to N-cadherin, osteopontin or VCAM-1 (Figure 6.8).

Adhesion to N-cadherin and osteopontin was also tested in LTC-IC adhesion assays, to study the behaviour of more primitive progenitors and the effect of culture with cytokines (Figure 6.9). N-cadherin and osteopontin specific LTC-
Figure 6.6: Timecourse of changes in adhesion with cytokine activation. Adhesion assays were performed on hCD34+ cells that had been cultured in cytokines for 0, 16, 24, 48 and 72 hours, and percent progenitor adhesion quantified. Each donor was tested in triplicate, Mean ± SEM, n=3. P<0.001 for difference between 0 and 16 hours for all three ligands, Student’s t-test.
In order to demonstrate that the observed adhesion on N-cadherin, osteopontin and VCAM-1 was specific to the ligands, the cells were pre-treated with an N-cadherin antagonist peptide (CHAVC), anti-CD44 or anti-CD49d blocking antibody, respectively, before performing parallel adhesion assays with non-treated cells. Significant inhibition effect is demonstrated in all cases (p<0.05, Student’s t-test), for both fresh and cytokine activated cells after specific blockade, in comparison to baseline levels.

Figure 6.7: Receptor blockade in adhesion. In order to demonstrate that the observed adhesion on N-cadherin, osteopontin and VCAM-1 was specific to the ligands, the cells were pre-treated with an N-cadherin antagonist peptide (CHAVC), anti-CD44 or anti-CD49d blocking antibody, respectively, before performing parallel adhesion assays with non-treated cells. Significant inhibition effect is demonstrated in all cases (p<0.05, Student’s t-test), for both fresh and cytokine activated cells after specific blockade, in comparison to baseline levels.
Figure 6.8: Isotype and scrambled peptide treatments in adhesion. Fresh cells were treated with isotype controls for the anti-CD44 (rat IgG2b) and anti-VLA-4 (mouse IgG1) antibodies, a scrambled peptide control (Ac-CHGVC-OH) for the N-cadherin blocking peptide or PBS (untreated). Specific progenitor adhesion was measured. All differences between treated and untreated cells are non-significant. Mean ± SEM is shown for all conditions, n=3.
Figure 6.9: Specific LTC-IC adhesion to N-cadherin and osteopontin. Specific adhesion of fresh and cultured LTC-IC to N-cadherin and osteopontin. Baseline adhesion (to PBS) was deducted from the adhesion to each ligand, to obtain percentage specific adhesion. Individual donors are shown, NS, paired Student’s t-test.
IC adhesion was reduced after culture in cytokines. Non-cultured LTC-IC had mean adhesion to N-cadherin of 15.40 ± 5.728% which was decreased to 4.840 ± 2.190% after cytokine culture. Similarly, LTC-IC adhesion to osteopontin was 13.14 ± 7.056% but was decreased to 3.170 ± 0.5689% after culture in cytokines. Due to the high variability of the results, however, these differences did not reach statistical significance and this assay was not used to investigate adhesion to any of the other ligands.

### 6.2.3 Pathway confirmation in the attachment model

The experiments described above suggest that exposure to cytokines induces an adhesion defect related to specific ligands that are present in the BM niches. Next, the *ex vivo* attachment model was used to confirm the relevance of these observations in a more physiologically relevant system. Fresh (uncultured) hCD34\(^+\) cells were treated with specific blocking antibodies or peptide prior to infusion into the rat bones (as described in Chapter 5). As shown in Figure 6.10 blockade of CD44, VLA-4 and N-cadherin on uncultured hCD34\(^+\) progenitors significantly reduced the levels of attachment in the *ex vivo* BM model. N-cadherin blockade reduced the number of attached progenitors from 635.9 (454.3-954.2) to 262.8 (25.48-535, p<0.005, Mann-Whitney test). Similarly CD44 blockade reduced progenitor attachment from 558.2 (306.0-731.7) to 336 (168.8-422.1, p<0.05) and VLA-4 blockade from 446 (491.6-754.6) to 270.8 (260.4-497, p<0.005). Moreover, combined blockade of all three receptors did not further reduce the attachment levels of fresh cells (Figure 6.11). The specificity of the blockade of each pathway was confirmed by the use of isotype control antibodies, or scrambled peptide in the case of N-cadherin (Figure 6.12). These observations confirm the importance of these receptor/ligand interactions in progenitor attachment in the BM.
Figure 6.10: Effect of receptor blockade on attachment of fresh hCD34+ cells in the ex vivo bone marrow model. Fresh progenitor attachment in the rat model was assessed. The infused cells were treated with the N-cadherin antagonist peptide, blocking antibodies for CD44 and VLA-4 or PBS. Data are shown for individual bones. *p<0.05, **p<0.01, Mann-Whitney test.
Figure 6.11: Combined receptor blockade on attachment of fresh hCD34^+ cells in the ex vivo bone marrow model. An N-cadherin antagonist peptide was used for N-cadherin and blocking antibodies for CD44 and VLA-4 or a combination of all three, as indicated. Untreated progenitor attachment is significantly higher (p<0.05, Mann-Whitney) than all the blocking treatments. All other combinations of the treated conditions, including combined blockade, are not significantly different from one another.
Figure 6.12: Effect of isotype control antibodies (for CD44 and VLA4 antibodies) and scrambled peptide (for N-cadherin peptide) in the ex-vivo BM model. Uncultured cells were incubated with N-cadherin antagonistic peptide (CHAVC) or a scrambled control peptide (CHGVC), anti-CD44 and anti-VLA-4 blocking antibodies or their isotype controls at the same conditions and concentrations. The cells were then infused in rat bones to evaluate progenitor attachment. The blocking antibodies and N-cadherin antagonistic peptide significantly reduced attachment in the BM in comparison to the controls. Analysis by Mann-Whitney, *p<0.05, **p<0.01.
6.2.4 Expression changes with cytokine activation

Altered attachment of HSPC may be related to changes in the surface expression of the adhesive receptors on these cells following cytokine activation. Therefore the next series of experiments were designed to examine the expression of the relevant receptors on hCD34^+ cells before and after culture with cytokines. Surface expression of all three receptors (N-cadherin, CD44 and VLA-4) increases following cytokine activation as is shown in the representative histograms by flowcytometric analysis (Figure 6.13).

To establish the kinetics of this upregulation, timecourse experiments were performed and surface expression was analysed both as the percentage of positive cells and the Mean Fluorescence Intensity (MFI) ratio, in comparison to isotype controls, for each receptor (Figure 6.14). Following cytokine culture expression of N-cadherin and VLA-4 (CD49d) is upregulated. Basal expression of CD44 is high in fresh cells (98 ± 0.5%) and thus does not significantly increase with cytokine activation. N-cadherin is expressed in only 3.4 ± 0.82% (Mean ± SEM) of fresh hCD34^+ cells but it is tripled within 48 hours and reaches 24.73 ± 4.13% after 72 hours of culture (Figure 6.14, top panel). Maximal increase in surface expression of N-cadherin per cell (as indicated by the MFI ratio) occurs at 48 hours of culture (Figure 6.14, top panel). VLA-4 is expressed on 54.59 ± 11.1% of the fresh hCD34^+ cells and increases to 91 ± 2.87% of the CD34^+ population within 24 hours of exposure to cytokines (Figure 6.14, top panel). Surface expression of the receptor (MFI ratio) rose gradually over the 72 hours in culture (Figure 6.14, bottom panel).

Expression of the respective transcripts was also investigated for fresh and cultured cells (Figure 6.15). There was a 2-fold increase in VLA-4 mRNA expression in cells that had been cultured in cytokines compared to uncultured cells. N-CADHERIN and CD44 mRNA expression, however, was
Figure 6.13: Expression of adhesion receptors on fresh and cultured hCD34$^+$ cells. Representative FACS plots of N-cadherin, CD44, and CD49d. Profiles for fresh cells are shown in grey, activated cells in black and isotype control with a dotted line.
Figure 6.14: Timecourse in expression of adhesion receptors with cytokine culture. Top panel: Percentage of hCD34$^+$ cells positive for N-cadherin, CD44 and CD49d at 0, 24, 48 and 72 hours post cytokine activation. Bottom panel: Fold difference in Mean Fluorescence Intensity (MFI), compared to controls stained with isotype-matched antibodies, at 0, 24, 48 and 72 hours post cytokine activation. Mean ± SEM, n=5.
Figure 6.15: MRNA expression of *N-CADHERIN, CD44 and VLA-4*. The mRNA expression of cytokine activated hCD34+ cells in comparison to fresh cells was analysed by Q-PCR. Fold difference of receptor mRNA expression (grey) in relation to fresh cell expression (black) is shown. Mean ± SEM, n=4.
moderately reduced after the 3-day culture in cytokines. *N-CADHERIN* mRNA was reduced by 0.79 ± 0.03 compared to fresh cells and *CD44* to 0.53 ± 0.07 (Figure 6.15). Interestingly, while the changes in *CD44* and *VLA-4* occur gradually, *N-CADHERIN* is upregulated in the first 24 hours but is subsequently reduced to levels below those of fresh cells (Figure 6.16).

### 6.3 Discussion

This section of the work aims to dissect the cytokine-induced HSPC attachment defect that was suggested by the *in vivo* and *ex vivo* assays previously described (Chapter 4 and 5). A variety of ligands were selected for this study that have been implicated in the adhesion and retention of HSPC in the osteoblastic or vascular niches of the BM (section 1.6).

Freshly isolated HSPC demonstrated specific adhesion to N-cadherin, osteopontin, VCAM-1 and fibronectin. Progenitor adhesion to these ligands varied from 24% for osteopontin to 36% for VCAM-1 and was significantly higher than background levels, in all cases. After culture in cytokines specific adhesion to N-cadherin, osteopontin and VCAM-1 was significantly reduced whereas adhesion to fibronectin did not significantly change.

In regards to fibronectin adhesion, previous reports in MPB and CB cells have demonstrated fluctuating adhesion to this ligand, increasing for cycling cells and returning to baseline levels after completion of cell cycle (Giet *et al*, 2001; Huygen *et al*, 2002). These studies are in agreement with the data shown here, although the authors had not looked at progenitor adhesion.

Literature on VCAM-1 adhesion is contradictory as cytokine activation has been reported to increase (Foguenne *et al*, 2009), decrease (Huygen *et al*, 2002) or have no effect (Giet *et al*, 2001) on CD34+ cell adhesion to this ligand. These data are produced in a variety of assays and cell sources and have not quantified the CFC, factors that could explain the different results.
Figure 6.16: Timecourse in mRNA expression of \textit{N-CADHERIN}, \textit{CD44} and \textit{VLA-4}. mRNA was isolated from cells that had been cultured in cytokines for 24, 48 and 72 hours, as indicated. \textit{N-CADHERIN}, \textit{CD44} and \textit{VLA-4} expression was analysed in comparison to fresh cells by Q-PCR. Fold difference of receptor mRNA expression in relation to fresh cell expression is shown. Mean ± SEM is shown, n=3.
Here, in MPB progenitors we observed a significant reduction in the levels of specific adhesion of these cells to VCAM-1, which could be related to their attachment defect in the BM.

Other significant components of the endosteal niches, such as N-cadherin and osteopontin have not been studied in the context of cytokine activation. Here we observed a considerable decrease in progenitor adhesion to N-cadherin and osteopontin after culture in cytokines; especially for osteopontin, where cytokine activation reduced progenitor adhesion by half.

The clonogenic assays, which are used as readout, depict the adhesion levels of a functionally defined population of progenitor cells. This excludes the possibility that this reduction in adhesion is related to HSPC differentiation with culture. Moreover, the LTC-IC data (for N-cadherin and osteopontin) further support these observations. Finally, the changes in adhesion occur within 16 hours of exposure to the cytokine cocktail and before the cells have expanded (Figure 3.1).

Reduction in HSPC adhesion to these ligands may be one of the underlying factors that prevent the long-term retention and engraftment of cultured hCD34⁺ cells in vivo, especially after intraosseous injection, when the non-specific homing process is by-passed. Blockade of N-cadherin, CD44 and VLA-4 on fresh hCD34⁺ cells significantly inhibited progenitor attachment in the rat model. Interestingly, combined blockade of all three pathways did not further decrease attachment, which underlines the individual importance of each of these adhesive interactions in HSPC attachment. To better describe this, future work could test the effects of N-cadherin, VLA-4 and CD44 blockade in long-term engraftment of fresh hCD34⁺ cells after IB injection.

It could be argued that alterations in N-cadherin, osteopontin and VCAM-1 binding may simply result from downregulation of the relevant receptors with culture. Results here, however, demonstrated that surface expression of N-
cadherin, CD44 and VLA-4 on hCD34+ cells is retained or increases for the population as a whole with culture. Similar results on VLA-4 (Yamaguchi et al, 1998; Huygen et al, 2002) and CD44 (Becker et al, 1999; Berrios et al, 2001) expression on cycling cells have been previously reported. Interestingly, high expression of VLA-4 has been correlated with reduced engraftment potential, in murine HSC (Orschell-Traycoff et al, 2000). The same group observed no effect on engraftment from CD44 expression differences. Moreover, the mRNA data do not support the receptor downregulation hypothesis. The reduction in adhesion is evident in the first 16 hours after exposure to cytokines but by the first 24 hours no downregulation of any of the related receptor transcripts was observed.

Collectively these observations suggest that cytokines induce functional changes in the related receptors which affect their binding to specific ligands. Post-translational modification patterns have been shown to affect CD44 binding to its ligands (Lesley et al, 1993, 1995). These conformational changes for CD44, as well as for N-cadherin and VLA-4 could be caused through a variety of pathways. Integrin adhesion is often affected merely by surface clustering without any other expression or affinity changes (Danilov & Juliano, 1989; Faull et al, 1993; Jakubowski et al, 1995; Stewart et al, 1996).

One possible mechanism that could be altering receptor distribution is the clustering of lipid rafts on the surface of HSC in response to cytokine stimulation. Lipid raft clustering after cytokine activation has been reported for murine progenitors (Yamazaki et al, 2006). Such clustering affects the interaction and function of any lipid raft-associated receptors (Wysoczynski et al, 2005; Jahn et al, 2007; Lee et al, 2010a). A similar phenomenon could be affecting the function of CD44, N-cadherin and VLA-4 in MPB CD34+ cells after their exposure to cytokines. Further work in this context could investigate and modulate this and other possible contributing factors.
B: Modulations to improve the function of cultured HSPC

7 CD26 inhibition in homing and engraftment

7.1 Introduction

7.1.1 CD26 function

CD26/dipeptidylpeptidase IV is a 110-kDa membrane-bound protease that is expressed on various cell types including the apical surface of epithelial cells (Tsugiki et al., 1998), connective tissue (Iwaki-Egawa et al., 1998) and HSPC (Ruiz et al., 1998; Christopherson et al., 2002). Its soluble form retains its catalytic activity (Pereira et al., 2003) and is often associated with extracellular matrix (Levy et al., 1999) while it has been shown to bind directly to collagen (Löster et al., 1995). Its catalytic action is achieved by cleavage of dipeptides from the N-terminus of polypeptides that contain the X-Pro or X-Ala dipeptide (Christopherson et al., 2002; Lambeir et al., 2003). This rather common recognition pattern results in a wide range of potential substrates (such as cytokines GM-CSF, G-CSF, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-8, IL-10, IL-13, and EPO) although not all of these candidates appear to be targets of the exopeptidase (Hoffmann et al., 1993). SDF-1, however, is a direct target of CD26 (Lambeir et al., 2001). The N-terminus of chemokines, which is targeted by CD26 is essential for activation of and binding to seven-transmembrane G-protein linked chemokine receptors, a function that is abrogated by CD26 catalysis (Oravecz et al., 1997; Van Coillie et al., 1998; Lambeir et al., 2001). SDF-1 in particular, when cleaved by CD26, loses its chemotactic effect on CXCR4 expressing cells and indeed acts as an antagonist resulting in reduced SDF-1 directed migration (Christopherson et al., 2002, 2003a). Biologically this may lead to the generation of gradients or specific
microenvironments with enzymatic activity (Löster et al, 1995). It has also been shown to affect fibronectin mediated cell movement and adhesion (Miyamoto et al, 1987) and aminoacid salvage (Frohman et al, 1989).

7.1.2 CD26 inhibition; diprotin A

Based on the described roles of CD26, inhibition of its function has been attempted in several research areas as well as in stem cell transplantation. A few molecules have been discovered that are able to bind to and inhibit CD26 function, such as L-Val-L-boroPro (Günther et al, 1995), valine-pyrrolidide (Deacon et al, 1998) and gliptins, such as the recently described alogliptin (Feng et al, 2007). The most well studied molecule however, especially in stem cell research, has been diprotin A.

Diprotin A is a three aminoacid peptide (ILE-PRO-ILE) that was first described as a CD26 inhibitor in 1984 (Umezawa et al, 1984). Diprotin A functions as a competitive antagonist for CD26, because the tripeptide acts as substrate for the peptidase. Binding of diprotin A to CD26 is reversible (Rahfeld et al, 1991). Twelve years after its discovery it was first used as a pre-treatment on CD34+ CB cells and it increased their SDF-1 driven migration (Christopherson et al, 2002). Soon after, the same group also demonstrated that G-CSF mobilisation of HSPC is dependent on CD26 as when the exopeptidase is inhibited (Christopherson et al, 2003a) or knocked out (Christopherson et al, 2003b) circulation of haemopoietic progenitors is significantly decreased. Syngeneic transplantation experiments in mice showed that pre-treatment of BM Sca-1+/lin- with diprotin A could increase both their short-term homing and their long-term engraftment while it also enhanced their competitive repopulation and engraftment after secondary transplantation, compared with control cells (Christopherson et al, 2004). In these studies the HSPC were pre-incubated with diprotin A at 5mM (170.7mg/ml), for 15 minutes, at 37°C (standard treatment).
A year later Guo and colleagues reported that treatment of mouse embryonic stem cells with diprotin A enhanced their SDF-1 directed migration and long-term survival (Guo et al, 2005). The pre-treatment of the cells in this study was for 30 minutes at 37°C and at a much lower concentration of 5mg/ml Diprotin A. In a mouse congenic transplantation model, retrovirally transduced haemopoietic progenitors were shown to have increased levels of engraftment after treatment with diprotin A, compared to control (Tian et al, 2006). In these experiments CD26 blockade was achieved with the standard treatment but the cells were washed extensively before infusion into pre-irradiated mice (Tian et al, 2006).

Using CD34$^+$ and CD34$^+$/CD38$^-$ CB cells Christopherson and colleagues showed that culture with G-CSF and GM-CSF increases surface expression of CD26, which they associate with the reduced ability of these cells to migrate towards an SDF-1 gradient. Standard diprotin A pre-treatment restored the migratory function of these cells (Christopherson et al, 2006). The same group later showed that diprotin A pre-treatment can also increase long-term engraftment of CD34$^+$ or Lin$^-$ CB cells (Christopherson et al, 2007). Enhanced engraftment of diprotin A treated CD34$^+$ CB cells was also reported by the Broxmeyer group in the same year (Campbell et al, 2007). In a different xenotransplantation setting, human BM cells were shown to have increased homing to the foetal liver, long-term engraftment and better performance in competitive repopulation assays, after in utero HSCT of diprotin A treated cells in pregnant BALB/c mice (Peranteau et al, 2006). In this case the incubation was carried out at room temperature, with 5mM diprotin A and the cells were washed prior to infusion.

Although the protocols followed in the studies above varied slightly in the temperature, washes, incubation time and concentration, the same basic principle was followed (of *ex vivo* treating the cells before infusion). When this protocol was applied to G-CSF MPB CD34$^+$ cells, however, no effect was
observed on SDF-1 migration or long-term engraftment in irradiated immunodeficient mice (at either 5mM or 20mM diprotin A) (Kawai et al, 2007). On the contrary, when the authors infused diprotin A intravenously into the mice, long-term engraftment was markedly increased suggesting that inhibition of CD26 on the transplanted cells alone is not enough to have an effect on MPB CD34+ cell engraftment. Support for this notion was found in the observation that, after irradiation, the BM stroma expresses high levels of enzymatically active CD26 (Kawai et al, 2007). Diprotin A infusion prior to cell transplantation was also shown to significantly increase engraftment in competitive repopulation assays, in B6BoyJ mice (Broxmeyer et al, 2007). In this protocol the recipient animals were infused with diprotin A (5µM/recipient) or PBS, twice a day, for 2 days prior to cell infusion. Pre-treatment of recipient mice with diprotin A resulted in enhanced HSC engraftment in competitive repopulation assays. Furthermore, in secondary transplantation experiments, engraftment of the HSC obtained from the diprotin A treated animals was significantly higher (Broxmeyer et al, 2007).

In contrast to these studies, a recent report using a CD45.2/CD45.1 congenic mouse model, showed no advantage in pre-treating the cells with diprotin A before transplantation in PB, BM and spleen chimerism. The authors also attempted IV administration of diprotin A (or sitagliptin) neither of which increased engraftment levels of BM cells, although a low number (5) of animals was used (Schwaiger et al, 2012).

The effect of CD26 inhibition in the context of cytokine activation (for gene transduction) of HSPC has been investigated in murine lin- BM cells. *Ex vivo* culture of murine HSC with SCF and IL-6 (followed by overnight transduction with γ-retroviral supernatant) increased CD26 expression (Wyss et al, 2009) and significantly reduced long-term engraftment of the transplanted cells (Goebel et al, 2002). Similar data have also been published on CB HSC (Christopherson et al, 2006). Based on these observations the authors
attempted to increase the homing and engraftment of \textit{ex vivo} cultured HSC by blocking CD26 with diprotin A. Interestingly, they observed a significant increase in homing and engraftment of fresh HSC after CD26 blockade but a similar effect was not seen in cells that have been cultured in cytokines either for 3 days or overnight (Wyss \textit{et al}, 2009).

### 7.1.3 Chapter aims

Overall, CD26 inhibition can enhance the migration \textit{in vitro} and engraftment and survival of HSC \textit{in vivo}, although this effect appears to be dependent on the protocol and more importantly the source of the cells used. Freshly isolated MPB HSC express low/absent levels of CD26 and this may underlie their greater migratory and homing potential compared with steady state BM cells (Bonig \textit{et al}, 2007). Cultured cells express higher levels of CD26 but have reduced homing and engraftment. CD26 inhibition may, therefore, represent a novel approach to increase the efficacy and success of HSCT, especially under conditions of limited donor cell yield. The little data that exists on G-CSF MPB CD34$^+$ cells, however, suggests that CD26 inhibition in the marrow milieu and not only on the infused cells themselves, is required to increase long-term engraftment. So far, no data has been published on the effect of diprotin A treatment in cytokine cultured MPB HSPC.

The aim of this part of the work was to describe the role of SDF-1 as modifier of adhesion and as chemoattractant and to investigate whether the \textit{in vitro} and \textit{in vivo} properties of fresh and cytokine activated MPB CD34$^+$ cells can be modulated by CD26 inhibition.
7.2 Results

7.2.1 CD26 expression

*Ex vivo* culture of murine and CB HSPC with cytokines has been shown to increase CD26 expression (Christopherson *et al.*, 2006; Wyss *et al.*, 2009). No such data has been reported for cytokine activated MPB HSPC. CD26 expression was initially assessed on fresh and cultured MPB CD34+ cells. The proportion of freshly isolated CD34+ cells that express CD26 demonstrated great variability between different donors. Expression levels for every individual donor, however, increased with culture. Freshly isolated HSPC were cultured in the standard cytokine mix for 72 hours and surface expression of CD26 was tested at 0, 24 and 72 hours by FACS. As seen in Figure 7.1 both the percentage positive cells and the protein expression per cell (MFI ratio) increased after culture in cytokines. In fresh/uncultured cells, 7.55 ± 2.243% were positive for CD26 and that percentage increased to 21.93 ± 4.316% after 72 hours of culture in cytokines (n=5, p<0.05, paired Student’s t-test). This increased expression is in agreement with observations made with other HSPC cell sources and could have a negative impact on homing and engraftment of cultured hCD34+ cells as it may reduce SDF-1 availability in the vicinity of transplanted cells.

It is important to note that CXCR4 protein and mRNA expression also increased with exposure of MPB HSPC to cytokines, as discussed in section 3.2.3 which may, to some degree, counteract the CD26 increase in cultured cells.

7.2.2 SDF-1 effect on adhesion

In addition to the many other described roles of SDF-1 in HSPC homing and engraftment, the importance of the availability of the chemokine at the sites of HSPC adhesion has been proposed. SDF-1 has been shown to stimulate
integrin-mediated progenitor attachment on vascular endothelium (Peled et al, 1999a) which results in increased engraftment (Peled et al, 2000). Additionally, altered dependence on the CXCR4/SDF-1 axis after exposure to cytokines has been suggested by the Papayannopoulou laboratory (Bonig et al, 2006). Therefore the aim of the following experiments was to establish whether presence of SDF-1 would affect progenitor adhesion to niche ligands and to investigate any differences between fresh and cultured cells.

Adhesion assays were performed on N-cadherin, osteopontin, VCAM-1 and fibronectin in 100ng/ml SDF-1 or medium alone and progenitor adhesion was measured with colony assays. Any effect of SDF-1 on colony formation was excluded by seeding appropriate controls from the starting cell suspension. Background adhesion (to PBS) was moderately, non-significantly, decreased in the presence of SDF-1. It was observed, in both fresh and cytokine activated cells, that SDF-1 significantly increased specific adhesion of hCD34+ to N-cadherin, and osteopontin, while it reduced adhesion to VCAM-1. In the case of fibronectin, SDF-1 has a greater effect on the adhesion of fresh, compared to cytokine cultured hCD34+ cells, resulting in relatively lower adhesion in the latter (Figure 7.2).

These results suggest that the presence of SDF-1 at the time of HSPC lodgment would increase progenitor adhesion of fresh and cultured cells to the endosteal niche-ligands (N-cadherin and osteopontin) and may attenuate the cytokine-induced defect. Based on these observations it can be hypothesised that CD26 inhibition, which prevents SDF-1 degradation, could increase SDF-1 availability and therefore adhesion of HSPC to these niche ligands potentially increasing long-term engraftment. The following experiments aimed to test this hypothesis and investigate the effect of CD26 inhibition in the behaviour of fresh and cultured cells in vitro and in vivo.
Figure 7.1: Timecourse of CD26 expression. Freshly isolated HSPC were cultured in the standard cytokine cocktail for 72 hours. A) Representative histograms of CD26 FITC staining as analysed by FACS, at 0, 24 and 72 hours. Isotype controls are shown in grey and CD26 staining in black. B) The percentage positive cells (top panel) and MFI ratio (bottom panel) are indicated separately for each donor, n=5.
Figure 7.2: The effect of SDF-1 on adhesion to ligands. Adhesion assays were performed in the presence of 100ng/ml SDF-1 (closed bars) or normal media (open bars) for fresh and cultured hCD34\(^+\) cells. *p<0.05, **p<0.01, Student's t-tests. The difference in adhesion between fresh and cultured HSPC in the presence of SDF-1 is non-significant for all four ligands. N=5 for N-cadherin and fibronectin, n=4 for VCAM-1 and n=3 for osteopontin.
7.2.3 Effect of CD26 inhibition on the adhesion of fresh and cultured MPB HSPC

Fresh and cultured progenitors were incubated for 15 minutes at 37°C, with diprotin A at 5mM (standard treatment) or control (PBS). The cells were then used in adhesion assays with N-cadherin, osteopontin and VCAM-1, without prior wash. The adhesion assays were performed in the presence or absence of SDF-1 (100ng/ml). Cytokine activation reduced adhesion to all three ligands whereas SDF-1 increased adhesion to N-cadherin and osteopontin but not to VCAM-1 (Figures 7.3, 7.4 & 7.5), as previously described.

Diprotin A pre-treatment did not have any additional impact on adhesion to N-cadherin either in the presence or absence of SDF-1. Notably, in cytokine activated cells, which have higher CD26 expression, diprotin A did not rescue the cytokine-induced reduction in adhesion to N-cadherin (Figure 7.3).

Diprotin A pre-treatment had different impact on HSPC adhesion to osteopontin. Adhesion of uncultured progenitors to osteopontin was significantly increased by CD26 inhibition (20.79 ± 2.858% vs. 38.54 ± 1.768% for pre-treated cells, p<0.01, Student’s t-test) (Figure 7.4). In the presence of SDF-1, adhesion to osteopontin was also increased (31.58 ± 3.386%). When the fresh, diprotin A treated cells, however, were allowed to adhere to osteopontin, in the presence of SDF-1, progenitor adhesion dropped to 3.643 ± 3.254% (Figure 7.4). Previous experiments have shown that adhesion to all ligands is decreased in the presence of high concentrations of SDF-1 (data not shown). The presence of SDF-1 in combination with diprotin A could have increased the local concentration of the chemokine to such levels that adhesion was, in this case, inhibited. In cytokine cultured cells adhesion increased in the presence of SDF-1 from 1.223 ± 0.3153% to 10.94 ± 0.7391% (p<0.01, Student’s t-test) but the
cytokine-induced adhesion defect was not rescued (Figure 7.4). Adhesion to osteopontin was unaffected by diprotin A pre-treatment.

Finally, as with osteopontin, diprotin A pre-treatment increased adhesion of fresh cells to VCAM-1. Untreated fresh HSPC had 72 ± 1.922% specific adhesion to VCAM-1 vs. 79.53 ± 0.5028% (p<0.05) for diprotin A treated cells (Figure 7.5). Diprotin A treated, fresh HSPC have similar adhesion to VCAM-1, in the presence of SDF-1 (77.01 ± 1.184%), and that was significantly higher than that of untreated cells, in the presence of SDF-1 (56.47 ± 5.469, p<0.01, Student’s t-test) (Figure 7.5). After culture in cytokines diprotin A pre-treatment had no effect on VCAM-1 adhesion and no additional changes were observed in the presence of SDF-1, for the treated cells (Figure 7.5).
Figure 7.3: The effect of SDF-1 and diprotin A on adhesion to N-cadherin. Adhesion assays were performed in the presence or absence of 100ng/ml SDF-1 as indicated, for fresh and cultured hCD34+ cells. The cells were pre-treated with diprotin A (stripes) or PBS (no stripes). Mean ± SEM is shown, *p<0.05, **p<0.01, Student’s t-test, n=3.
Figure 7.4: The effect of SDF-1 and diprotin A on adhesion to osteopontin. Adhesion assays were performed in the presence or absence of 100ng/ml SDF-1 as indicated, for fresh and cultured hCD34<sup>+</sup> cells. The cells were pre-treated with diprotin A (stripes) or PBS (no stripes). Mean ± SEM is shown, **p<0.01, Student’s t-test, n=3.
Figure 7.5: The effect of SDF-1 and diprotin A on adhesion to VCAM-1. Adhesion assays were performed in the presence or absence of 100ng/ml SDF-1 as indicated, for fresh and cultured hCD34+ cells. The cells were pre-treated with diprotin A (stripes) or PBS (no stripes). Mean ± SEM is shown, *p<0.05, **p<0.01, Student’s t-test, n=3.
7.2.4 Effect of CD26 inhibition on the migration of fresh and cultured MPB HSPC

Cytokine culture of MPB CD34+ cells has been shown to increase random and SDF-1 directed migration (Yong et al, 1998 and section 3.2.6) which may lead to the non-specific tissue localisation observed after expansion (Ahmed et al, 2004). Here the effect of CD26 inhibition on random and SDF-1 directed progenitor migration was investigated. Fresh or 72 hours cultured hCD34+ cells were pre-incubated with diprotin A (standard treatment) and used in migration assays as previously described. As a control some of the cells were pre-treated with the CXCR4 inhibitor AMD3100 or with PBS (control), at 5µg/ml, for 30 minutes, at 37 °C.

As shown in Figure 7.6 fresh cells have low levels of random migration and they respond to the SDF-1 gradient. Activated cells have high levels of both random and SDF-1 directed migration. CXCR4 blockade (with AMD3100) reduced progenitor migration to baseline levels for fresh and cultured cells. That is particularly interesting in the case of the ex vivo expanded cells where random migration was already increased (1.658 ± 0.2561% for fresh cells vs. 15.03 ± 1.781% for cultured cells). This increase in random migration after cytokine culture appears to be CXCR4 independent, as it was not further decreased after AMD3100 treatment.

CD26 inhibition has no effect on random or SDF-1 directed migration of non-cultured progenitors. In cultured cells diprotin A treatment reduced SDF-1 directed migration from 44.17 ± 2.397% to 36.49 ± 3.578% but that difference did not reach statistical significance (Figure 7.6). Overall CD26 inhibition with
Figure 7.6: Progenitor migration with CD26 and CXCR4 inhibition. Migration of fresh and cytokine activated hCD34+ progenitors, as assessed by colony assays on the migrated cells. SDF-1 at 100ng/ml was placed in bottom chamber of Transwells as indicated. The cells were pre-treated with 5mM diprotin A or 5µg/ml AMD3100 (or PBS/untreated) as indicated and inhibitors were present throughout. Mean ± SEM, *p<0.01, **p<0.0001, n=9. Each donor was tested in duplicate.
diprotin A did not significantly affect progenitor migration, either before or after culture with cytokines.

**7.2.5 Effect of CD26 inhibition on homing to the BM**

Previous reports on CB (Christopherson *et al*, 2004) and murine (Wyss *et al*, 2009) HSPC have indicated an increase in short-term homing after *ex vivo* treatment of the cells with diprotin A. No similar data exist on MPB CD34+ cells especially in the context of cytokine activation.

We first aimed to investigate whether blockade of CD26 on the HSPC would improve the levels of homing after cytokine activation. Fresh and cultured hCD34+ cells were pre-treated with diprotin A (standard treatment) or PBS and infused at a dose of 2x10^6 cells/animal to sublethally irradiated β2m/NOD-SCID mice. Progenitor homing was quantified 20 hours later, as described in section 2.3.4.1. As shown in Figure 7.7 *ex vivo* diprotin A treatment significantly increases the homing of fresh progenitor cells to the BM (7.380%, range 1.110%-14.74% for untreated cells vs. 23.42%, range 3.907%-34.16% for diprotin A treated cells, p<0.05, Mann-Whitney test), which is in agreement with the aforementioned reports in other cell sources.

Cytokine culture reduces progenitor homing, as previously reported (section 3.2.5). *Ex vivo* treatment with diprotin A increased the homing of cultured cells from 0.7296% (0.29%-1.672%) to 1.086% (0.7598%-2.389%). Despite the fact that the difference between the treated and untreated cultured cells is statistically significant (p<0.05, Mann-Whitney test), the effect size is small and generally the cytokine-induced defect in homing was not rescued by CD26 inhibition on the expanded cells (Figure 7.7).

Previous work on MPB hCD34+ engraftment in the setting of CD26 inhibition suggests that IV infusion of diprotin A (instead of *ex vivo* treatment) can have an impact on the resulting engraftment (Kawai *et al*, 2007). Based on this
Figure 7.7: Effect of 

ex vivo diprotin A treatment on homing. Homing of fresh and cytokine cultured hCD34⁺ progenitors. Cells (2x10⁶ /animal) were injected by tail vein into sublethally irradiated β₂m/NOD-SCID mice with and without ex vivo Diprotin A pre-treatment. Homing to the BM was assessed with clonogenic assays (using human-specific cytokines) performed on cells obtained from long bones at 20 hours post-transplantation. Data are presented as % of infused progenitors as described in Chapter 2. Values are given for individual animals, with medians indicated. Statistical analysis by Mann-Whitney test, *p<0.05, ***p<0.001.
report we investigated whether *ex vivo* treatment and simultaneous IV infusion of diprotin A (with the cells) would rescue the homing of cultured progenitors. In addition to the pre-incubation with diprotin A, treated cells were infused with 200µl of diprotin A at 20mM. Due to reduced animal availability at the time, relatively older animals were initially used for these experiments (12-21 weeks old). Overall, the homing levels achieved in this set of experiments were lower than previously seen in younger animals (Figure 7.8). Despite that, cytokine activation reduced the levels of homing by half (0.8782%, range 0.4107%-1.171% for fresh vs. 0.4143%, range 0.1475%-0.6660% for cultured cells, p<0.01 by Mann-Whitney test).

The combined diprotin A treatment (*ex vivo* incubation and *in vivo* infusion) had no significant effect on the homing of fresh progenitors. After cytokine activation, however, this protocol restored the homing of the expanded cells to levels equivalent to those achieved with fresh cells (Figure 7.8). The diprotin A effect on cultured cells was statistically significant (0.4143%, range 0.1475%-0.666% for cultured, untreated cells vs. 0.8661%, range 0.3075%-1.427% for cultured, diprotin A treated cells, p<0.001 by Mann-Whitney test). In the context of diprotin A treatment, cytokine culture had no significant effect on homing.

The age of the animals used in this series of experiments appeared to have an effect on the levels of homing achieved. This could be related to the appearance of B and T-cell clones, as discussed in section 1.2.2.2.1. The same dose of fresh cells achieved lower levels of short-term homing in older animals. This was confirmed by linear regression analysis (Figure 7.9) which indicated that animal age correlates negatively with the levels of homing to the BM (p<0.05).

As the combined diprotin A protocol (standard *ex vivo* treatment and *in vivo* infusion) gave such encouraging results in the homing of cultured HSPC to
Figure 7.8: Effect of ex vivo treatment and in vivo diprotin A infusion on homing (older animals). HCD34+ cells (2x10^6/animal) were injected by tail vein into sublethally irradiated β2m/NOD-SCID mice. Where indicated the cells were ex vivo treated with diprotin A (5mM, 15 minutes, 37°C) and 200µl diprotin A (at 20mM) was injected to the animals with the cells IV. Homing to the BM was assessed with clonogenic assays (using human-specific cytokines) performed on cells obtained from long bones at 20 hours post-transplantation. Data are presented as % of infused progenitors as described in Chapter 2. Values are given for individual animals, with medians indicated. Statistical analysis by Mann-Whitney test, **p<0.01, ***p<0.001.
Figure 7.9: The impact of animal age on homing. Fresh hCD34+ cells (2x10^6 /animal) were injected by tail vein into sublethally irradiated β2m/NOD-SCID mice aged between 12 and 21 weeks. Homing to the BM was assessed with clonogenic assays (using human-specific cytokines) performed on cells obtained from long bones at 20 hours post-transplantation. Data presented for individual animals, analysis by linear regression, p=0.0135.
older animals the experiment was repeated using younger animals (6-8 weeks old). In this context the combined diprotin A treatment increased the homing of fresh progenitors (3.548%, range 2.410%-6.612% for untreated vs. 6.321%, range 5.041%-6.669% for fresh diprotin A treated HSPC). However, this difference did not reach statistical significance (Figure 7.10). Cytokine culture significantly reduced progenitor homing, as previously seen (3.548% for fresh vs. 0.8684% for cultured cells). Finally, the combined diprotin A protocol significantly increased cultured progenitor homing (0.8684%, range 0.06254%-1.254% for cultured untreated HSPC vs. 2.581%, range 1.473%-3.813% for cultured diprotin A treated HSPC, p<0.001). The levels of cultured progenitor homing achieved with this protocol were similar to those achieved by fresh untreated cells (Figure 7.10). In these animals, however, the cytokine-induced defect in homing persisted in the context of the combined diprotin A protocol; cytokine-cultured, diprotin A treated cells have significantly lower levels of homing in comparison to their fresh counterparts (6.321% for fresh, diprotin A treated cells vs. 2.581% for cultured, diprotin A treated cells, p<0.01).

7.2.6 Effect of CD26 inhibition on BM attachment

In order to further investigate the effect of CD26 inhibition on the resulting attachment of HSPC in the BM microenvironment, fresh and cultured hCD34+ cells were treated with diprotin A and infused into rat femurs and tibiae ex vivo, utilising the attachment model described in Chapter 5.

Ex vivo treatment with diprotin A combined with in vivo infusion was shown to have maximal impact on HSPC homing to the BM after cytokine culture. Hence, the conditions of this protocol were replicated with the ex vivo model to assess the effect of CD26 blockade on HSPC attachment. The infused cells were treated with diprotin A ex vivo as previously described (standard treatment). To replicate the in vivo conditions after infusion of diprotin A,
Figure 7.10: Effect of ex vivo treatment and in vivo diprotin A infusion on homing (younger animals). Cells (2x10$^6$/animal) were injected by tail vein into sublethally irradiated β2m/NOD-SCID mice. Where indicated the cells were ex vivo treated with diprotin A (5mM, 15 minutes, 37°C) and 200µl diprotin A (at 20mM) was also infused with the cells IV. Homing to the BM was assessed with clonogenic assays (using human-specific cytokines) performed on cells obtained from long bones at 20 hours post-transplantation. Data are presented as % of infused progenitors as described in Chapter 2. Values are given for individual animals, with medians indicated. **p<0.01, ***p<0.001, Mann-Whitney test.
assuming that the young animals have a circulation volume of approximately 2ml, in which 200µl of 20mM diprotin A was injected, the diprotin A levels in the blood would be approximately 2mM. Soon after injection, concentration of the peptide would reach the same levels in the BM. Based on this hypothesis, the ex vivo diprotin A treated cells were infused in the BM with diprotin A at 2mM. After incubation for 90 minutes, HSPC attachment was measured as previously described (section 5.2.3).

Cytokine culture (for 72 hours) significantly decreased the levels of attachment in the BM as has been previously discussed (section 5.3.3). In this series of experiments, the number of attached progenitors was reduced from 946.5 (558.9-1630) for fresh cells, to 524.3 (174-796.4) after cytokine culture. Diprotin A treatment reduced the attachment of both fresh as well as cultured progenitors but these differences were not statistically significant. Fresh HSPC attachment after diprotin A treatment was 672.4 (269.4-2647) while after cytokine culture the same treatment reduced attachment to 390.8 (52.34-798) (Figure 7.11). Diprotin A treatment had no effect on the cytokine-induced loss of attachment, in this model. Overall, these data support the adhesion assay results and indicate that diprotin A does not increase the HSPC attachment in the BM, especially for cultured progenitors.

### 7.2.7 CD26 inhibition in engraftment

CD26 inhibition has been shown to improve long-term engraftment of CB and murine progenitors (Christopherson et al, 2004; Peranteau et al, 2006; Tian et al, 2006; Christopherson et al, 2007; Campbell et al, 2007). Here the same protocol was used to test its effect on long-term engraftment of MPB CD34+ cells before and after a 3-day culture in the standard cytokine mix. As shown in Figure 7.12, ex vivo treatment of the infused cells with diprotin A had no effect on the resulting engraftment (in NOD/SCID mice) of either the fresh or the cytokine cultured CD34+ cells.
Figure 7.11: Effect of diprotin A on CFC attachment in the ex vivo model. Attachment of fresh and cytokine activated hCD34+ progenitors with and without CD26 inhibition. Cells (0.5x10^6 /ml) pre-treated with diprotin A (5mM) or PBS were infused in the rat bones with diprotin A (2mM) or PBS. Attachment in the BM was assessed from clonogenic assays (using human-specific cytokines) as described in Chapter 5. Data given for individual bones, with medians indicated. *p<0.5, ***p<0.001, statistical analysis by Mann-Whitney test.
Figure 7.12: Engraftment after IV infusion of HSPC with prior CD26 inhibition ex vivo.

Fresh and cultured hCD34+ cells were infused IV at doses of 5, 10 and 30x10^6 to sublethally irradiated NOD/SCID mice. Engraftment was assessed by FACS analysis of the BM of the animals at 8-10 weeks as detailed in General Methods. Data given for individual animals, with medians indicated. *p<0.05, Mann-Whitney test.
Ex vivo diprotin A treatment, combined with in vivo infusion was shown to increase HSPC homing (section 7.2.5). The next assays investigated whether this combined protocol can increase the long-term engraftment of cytokine cultured MPB CD34+ cells. These experiments were performed in β2m/NOD/SCID mice, due to reduced NOD/SCID animal availability. Although the β2m/NOD/SCID mice were also used for the homing assays, they introduce variability in the results due to the sex related differences discussed in section 2.3.4.2.3.

Cytokine activated cells were treated ex vivo with diprotin A or PBS, as previously described and infused in sublethally irradiated β2m/NOD/SCID mice with 200µl diprotin A (20mM) or PBS. Both the donors used, as well as the sex of the animals had an impact on the levels of MPB HSC engraftment achieved causing a significant baseline drift (Figure 7.13). The effect of the sex of the recipient animal on engraftment levels in β2m/NOD/SCID mice is shown in Figure 2.5. Despite this, the combined diprotin A protocol increased median engraftment in each animal group. Due to the low number of animals in the separate groups, however, these differences were not statistically significant. In order to summarise the data collected from these experiments engraftment was expressed as fold increase in comparison to the average untreated engraftment levels for each donor and sex. This normalisation reveals a moderate but significant impact of the diprotin A protocol on engraftment of cytokine cultured cells (Figure 7.14). Treated, cultured cells had 1.508-fold higher engraftment levels compared to their untreated counterparts (p<0.05, Mann-Whitney test).

It is important to note that diprotin A (in both protocols) had no impact on the differentiation of the engrafted cells as demonstrated by the expression of CD19 and CD33 in the BM of the recipient animals (Figure 7.15).
Figure 7.13: Variation in engraftment with the combined diprotin A protocol. Cultured hCD34+ cells were pre-treated ex vivo with diprotin A at 5mM (or PBS) and then infused IV with 100µl diprotin A at 20mM (or PBS) to sublethally irradiated β2M/NOD/SCID mice, at a cell dose of 2x10⁶/animal. Engraftment was assessed by FACS analysis of BM after 8-10 weeks. The donors used as well as the sex of the animals was found to have significant effects on the levels of engraftment achieved and are, therefore, shown separately. Females are shown in open circles and males in closed squares. Total engraftment for both donors used is shown in the top panels. Engraftment in female animals is shown in the middle panels and in male animals in the bottom panels.
Figure 7.14: Engraftment after IV infusion of HSPC with the combined diprotin A protocol. Cultured hCD34+ cells were pre-treated ex vivo with diprotin A at 5mM (or PBS) and then infused IV with 100µl diprotin A at 20mM (or PBS) to sublethally irradiated β2m/NOD/SCID mice, at a cell dose of 2x10^6/animal. Engraftment was assessed by FACS analysis of bone marrow at 8-10 weeks as detailed in General Methods. The levels of engraftment were normalised to the sex of the animals and the donor used and are expressed as fold increase in comparison to average untreated cell-engraftment. P<0.05, Mann-Whitney test.
Figure 7.15: Differentiation of engrafted cells with or without the diprotin A combined protocol. Cultured hCD34+ cells were pre-treated ex vivo with diprotin A at 5mM (or PBS) and then infused IV with 200μl diprotin A at 20mM (or PBS) into sublethally irradiated β2M/NOD/SCID mice, at a cell dose of 2x10^6/animal. Engraftment was assessed by FACS analysis of the BM of the animals after 8-10 weeks. HCD19PE and hCD33APC antibodies were used to demonstrate the multilineage nature of the hCD45+, engrafted cells. FACS plots are shown for CD45-gated cells from two representative animals; one that received untreated cells (top panel) and one that received the diprotin A treated cells (bottom panel).
7.3 Discussion

SDF-1 was shown here to increase adhesion of fresh and cytokine cultured progenitors to N-cadherin and osteopontin. This increase in adhesion could potentially minimise the cytokine-induced defect previously described. Cytokine activation, however, increased the expression of CD26 on these cells (section 7.2.1) which could lead to increased SDF-1 degradation and thus lower concentration of SDF-1 around the cells. Based on these observations it can be hypothesised that CD26 inhibition would increase SDF-1 availability in the microenvironment and thus enhance adhesion of the ex vivo expanded cells. Diprotin A treatment of cytokine activated cells prior to their use in adhesion assays, however, had no significant effects on their adhesion to N-cadherin, osteopontin and VCAM-1 in the presence or absence of SDF-1. In fresh cells diprotin A treatment mildly increased adhesion to these ligands, an effect which was especially pronounced for osteopontin. Adhesion of fresh diprotin A treated progenitors to osteopontin in the presence of SDF-1 was diminished, while no significant differences were observed for VCAM-1 and N-cadherin. These contradictory results could be related to the differential effect that varying concentrations of SDF-1 can have on the function of the receptors.

While these in vitro adhesion assays are very informative in terms of the interaction of specific receptors with their respective ligands it is difficult to accurately represent the SDF-1 concentrations present in vivo. For this reason in order to more efficiently study the effect of CD26 inhibition on HSPC attachment the diprotin A treatment was applied to cells that were used in the ex vivo attachment model.

Ex vivo treatment combined with in vivo infusion of diprotin A had no significant effect on the attachment of either fresh or cultured HSPC in the rat model and thus, did not rescue the cytokine-induced defect previously
described. Overall, summarising the in vitro and ex vivo assays presented here, CD26 inhibition with diprotin A did not enhance the attachment of cytokine cultured HSPC to BM elements.

SDF-1 plays a major role in HSPC transmigration and therefore upregulation of CD26 could affect the migratory behaviour of the expanded cells. CD26 inhibition with diprotin A, however, had no effect on the migration of fresh or cultured progenitors (section 7.2.4). This could be due to the concentration of SDF-1 used in these assays. If the SDF-1 concentration was saturating, the increase in SDF-1 levels via CD26 inhibition would be minimal and would not further increase HSPC migration. AMD3100, however, reduced the SDF-1 directed migration of fresh and cultured progenitors to baseline levels, confirming the role of CXCR4 in this pathway. Interestingly baseline migration increases significantly after culture with cytokines in a CXCR4 independent manner. The role of CD26 inhibition in trafficking and transmigration may be more physiologically represented in the in vivo homing assays.

Ex vivo treatment of MPB CD34+ cells with diprotin A significantly increased the homing of both fresh and cultured cells to the BM of irradiated immunodeficient mice (section 7.2.5). The increase was particularly evident in fresh HSPC, whereby median homing levels more than tripled after treatment with diprotin A (Figure 7.7). In cultured cells, however, a much smaller effect size was achieved with CD26 inhibition, which did not rescue the cytokine-induced defect. This observation suggests that inhibition of CD26 on the transplanted cells alone is not enough to overcome the homing deficiency of cultured cells. Similarly, when the same protocol was applied to long-term engraftment assays, where CD34+ cells were injected IV in immunodeficient mice, CD26 inhibition had no effect on either fresh or cultured cell engraftment (section 7.2.7). This could be because the increased non-specific homing of cultured cells was not overcome by CD26 inhibition. Additionally, the small increase in homing levels of cultured cells does not translate to
increased long-term engraftment as the attachment defect within the haemopoietic niches remains, as demonstrated in the *in vitro* adhesion assays and the *ex vivo* attachment model.

Cytokine activated HSPC may be more dependent on SDF-1 for their trafficking (Bonig *et al.*, 2006) and therefore it is possible that SDF-1 degradation by environmentally expressed CD26 (from BM stroma) is enough to reduce homing and engraftment to the BM. To test this possibility, diprotin A was infused IV with the transplanted cells (which had been previously *ex vivo* treated with diprotin A) and homing levels were quantified. This protocol significantly increased the homing of cytokine cultured progenitors to levels similar to those achieved by fresh, untreated cells (section 7.2.5). These levels, however, were significantly lower than those of fresh cells treated with the combined diprotin A protocol. This suggests that the cytokine-induced loss of homing is only partly rescued by CD26 inhibition, and additional pathways are involved.

To test if the increased homing observed with this protocol translated into improved engraftment, cultured cells were *ex vivo* treated and co-injected with diprotin A into sublethally irradiated mice and long-term engraftment was assessed at 8 weeks post-transplantation. This protocol was shown to increase long-term engraftment of fresh MPB CD34⁺ cells (Kawai *et al.*, 2007). In cytokine cultured MPB CD34⁺ cells, the same treatment increased median engraftment by 1.5-fold.

Due to reduced animal availability the combined diprotin A protocol effect on engraftment was tested on β₂m/NOD/SCID mice. After injection of 2x10⁶ CD34⁺ cells in these animals the levels of engraftment achieved were high for both fresh and cultured HSPC. The saturating cell number potentially masked the diprotin A effect on the long-term engraftment of cultured HSPC. This
issue should be addressed in further experiments, where limiting numbers of
diprotin A pre-treated or untreated cells are transplanted.

 Cultured HSPC have increased homing after *ex vivo* and *in vivo* diprotin A
treatment, but the reduced retention of HSPC in the BM may be unaffected by
this protocol. This is suggested by the effect of diprotin A on adhesion to BM
ligands, *in vitro* or attachment in the *ex vivo* model. The combined diprotin A
protocol, however, increased long-term engraftment of cultured HSPC, an
effect that could be more pronounced in situations were limited numbers of
HSPC are available. Further experiments, therefore, should confirm the
benefit of this treatment in a larger series of experiments, using more cell
doses and animals. Additionally, the importance of this pathway in
engraftment could be investigated using alternative means of blocking CD26,
such as RNA interference. If confirmed, this strategy could be exploited in
clinical HSCT where the CD34⁺ cell dose is limiting.
8 Neurotransmitters in HSC engraftment

8.1 Introduction

8.1.1 Clinical evidence of the interaction of the nervous system with haemopoiesis

A growing number of reports strongly support the role of the nervous system as the primary regulator of HSPC release from the BM and of haemopoiesis in general, by exercising direct and indirect control over haemopoietic stem cells.

Physiologic interactions between bone formation, the haemopoietic system and the nervous system have been reported in a variety of conditions over the past 150 years. Initial observations demonstrated that the nervous system has a direct impact on osteoblast function and subsequently, bone formation. Neurological disorders were associated with osteopenia and bone fragility (Gillespie, 1954). The connection between the nervous system and bone formation lead to the use of sympathetic ganglionectomy for the early treatment of unequal limbs in poliomyelitis patients (Barr et al, 1950). More evidence of this interaction came from patients suffering from “reflex sympathetic dystrophy”, a condition which leads to increased local adrenergic activity, as well as focal osteopenia and is managed with β-blockers (Schwartzman & McLellan, 1987).

Chronic and acute psychological stress was later linked to HSC mobilisation (Grisaru et al, 2001). It is a well described phenomenon that the immune function and the subsequent response to viral infections, chronic autoimmune diseases, and tumors is significantly affected by psychological stress (Kemeny & Schedlowski, 2007). This has been correlated to the impact psychosocial stress has on mature leukocytes (Kin & Sanders, 2006).
Moreover, spinal cord injury was reported to alter fracture healing (Freehafer & Mast, 1965) and has been linked to reduced immunity and haemopoiesis below the injury site (Iversen et al., 2000).

Further supporting this interaction between the nervous system and haemopoiesis, evidence shows that lithium treatments, which are used for bipolar disorders, cause neutrophilia (Murphy et al., 1971), which is accompanied by increased HSC circulation in vivo (Ballin et al., 1998) and granulocyte colony formation in vitro (Tisman et al., 1973). This is achieved through two separate mechanisms; lithium can downregulate CXCR4 expression on neutrophils which consequently alters their chemotaxis to SDF-1 and the resulting migration and trafficking (Kim et al., 2007). In a separate pathway lithium can block glycogen synthase kinase-3 (GSK-3) (Kast, 2008). GSK-3 inhibition leads to β-catenin upregulation which in turn promotes HSC proliferation (Reya et al., 2003). Additionally, GSK-3 blockade increases HIF-1 and subsequently the SDF-1/CXCR4 mediated migration and production of immature CD34+ cells (Kast, 2008).

8.1.2 Ex vivo evidence of the effect of neurotransmitters effect on HSPC

The early clinical evidence was soon supported by laboratory data which indicated that neurotransmitter signals drive the communication between the BM where the HSPC reside and the organ or tissue where they are required. Innervation of the BM was demonstrated in a variety of mammals. Myelinated and non-myelinated fibres were found to be in close contact with the endothelial layer of the BM sinuses and the origin of the arterioles (Calvo, 1968). Later studies, using immunohistochemistry, demonstrated that the periosteum can interact with neuromediators such as leptin, the vasoactive intestinal peptide and glutamate (Hohmann et al., 1986; Serre et al., 1999; Takeda et al., 2002).
At the same time *ex vivo* staining of cells of haemopoietic origin revealed expression of a wide variety of neuroreceptors.

### 8.1.2.1 Dopamine and dopamine receptors

Dopamine is one of the predominant neurotransmitters in the body with multiple functions. Dopamine is a catecholamine, thus it is formed by one amino group that is connected to an aromatic ring by a two-carbon chain and also contains a catechol group. All of the catecholamines arise from the aminoacid tyrosine at catecholamine-secreting neurons where dopamine is initially produced. Dopamine’s synthetic precursor is 3,4-dihydroxyphenylalanine (L-DOPA). Dopamine in turn, is the precursor to norepinephrine and epinephrine. As a neurotransmitter, dopamine is produced in specific areas of the brain including the hypothalamus and plays important roles in cognitive behaviour, voluntary movement, motivation, punishment and reward, sleep, mood, attention, working memory and learning (Schultz, 2002; Browman *et al.*, 2005; Flaherty, 2005; Matsumoto & Hikosaka, 2009).

Five separate dopamine receptors have been identified to date. They are metabotropic, G protein-coupled receptors and are divided in two families (the D$_1$-like receptor family and the D$_2$-like receptor family) based on their morphological characteristics such as the length of their -COOH terminal. The D$_1$-like receptors are D$_1$ and D$_5$ and the D$_2$-like receptors are D$_2$, D$_3$ and D$_4$ (reviewed in Civelli *et al.*, 1993; Vallone *et al.*, 2000). The transmembrane domains of D$_1$ and D$_5$ receptors are 80% identical whereas the D$_2$ and D$_3$ receptors have a 75% homology in their transmembrane domains, and the D$_2$ and D$_4$ receptors share a 53% identity. D$_1$ and D$_5$ receptors exhibit no pharmacological differences although dopamine has a higher affinity for D$_5$ than for D$_1$. Similarly, D$_3$ has 20 times higher affinity for dopamine in
comparison to D₂ and up to 100 times higher affinity for specific agonists such as quinpirole (Sokoloff et al., 1990).

The BM is innervated with noradrenergic sympathetic nerve fibres that can store and secrete dopamine (Mignini et al., 2003). This observation drove Spiegel and co-workers to look for expression of the dopamine receptors on HSPC. It was demonstrated that CD34⁺ cells from BM, MPB and CB express the dopamine receptors D₃ and D₅. Expression of the receptors was higher for the more primitive progenitors compared to the committed progenitors (for BM CD34⁺ cells) and G-CSF MPB had higher expression than CB progenitors (Spiegel et al., 2007). The effect of these receptors in haemopoiesis is further discussed in section 8.1.3.

8.1.2.2 Epinephrine, norepinephrine and adrenergic receptors

Epinephrine and norepinephrine (also called adrenaline and noradrenaline) are two related catecholamines, (products of dopamine) which are secreted by the adrenal medulla as well as sympathetic nerve fibres. The adrenal medulla exerts primarily epinephrine (80%) whereas the sympathetic nerve fibres synthesise mainly norepinephrine. Despite their homology, their roles in cardiovascular regulation differ. Both hormones can increase the rate and force of heart contraction; however, norepinephrine can constrict all blood vessels whereas epinephrine mainly constricts small blood vessels, while it dilates blood vessels in the skeletal muscles and the liver. Both catecholamines also have metabolic roles related to the circulation of fatty acids and the breakdown of glucose. Epinephrine in particular, is also involved in the “fight or flight” response (reviewed in Moore & Bloom, 1979).

Epinephrine and norepinephrine act by binding to a variety of receptors termed adrenergic receptors. The adrenergic receptors are also G protein-coupled, metabotropic receptors and they are divided in two main families; α (with subtypes α₁ and α₂) and β (with subtypes β₁, β₂ and β₃).
The α receptors in general, modulate constriction of the heart arteries, and veins, and also control the motility of smooth muscle in the gastrointestinal tract (Sagrada et al, 1987; Woodman & Vatner, 1987; Elliott, 1997). A1 antagonist prazosin was shown to increase circulation of granulocytes, platelets as well as the number of HSPC in the BM. Consistently, an α1-receptor agonist decreased the production of differentiated progenitor colonies in vitro (Maestroni & Conti, 1994). In agreement to these observations, norepinephrine administration in vivo was shown to rescue haemopoiesis from the non-cell cycle specific cytotoxic drug carboplatin by better maintaining the primary HSC population in the niches, but this protective effect was reversed with α1 receptor inhibition (Maestroni et al, 1997).

The β receptors have multiple roles in muscle function, as well as in the cardiovascular, gastrointestinal and respiratory systems. The subtypes β1 and β2 were first defined based on their relative affinities for epinephrine and norepinephrine (Stiles & Lefkowitz, 1984).

The β1 receptor is mainly expressed in the heart where it controls the rhythm and force of contraction as well as in the kidney where stimulation of the receptor leads to renin release.

The β2 adrenergic receptor is involved in the regulation of the respiratory, gastrointestinal, circulatory and reproductive systems while it also plays a variety of important roles in the bladder, liver, skeletal and smooth muscle, salivary glands, kidney and the immune system communication with the brain. The most significant clinical applications of β2 modulators so far, are in the respiratory system where bronchodilation is mediated by β2 receptors and in the reproductive system where β2 receptors control tocolysis (labour repression). Additionally, β2 receptor activation induces relaxation of the bladder, decreased motility in the gastrointestinal tract and hyperglycemia.
and lipolysis in the liver. In recent years, expression of the β_2_ receptor has been reported on osteoblasts and osteoclasts where it regulates bone formation and resorption (Moore et al., 1993; Kellenberger et al., 1998; Suzuki et al., 1999; Togari, 2002; Takeda et al., 2002). Interestingly haemopoietic progenitors from MPB and CB were also found to express the β_2_ adrenergic receptor, which prompted a variety of _in vivo_ experiments to elaborate the effect of adrenergic signaling in haemopoiesis, discussed in section 8.1.3 (Spiegel et al., 2007).

More recently a third β_3_ type (β_3_) adrenergic receptor was described, which promotes lipolysis, in adipose tissue and thermogenesis in skeletal muscle (Esler et al., 2003). Expression of the β_3_ receptor was detected in a subpopulation of stromal cells in the BM which drove further research into its involvement in HSPC mobilisation (Méndez-Ferrer et al., 2008). The authors describe that SDF-1 downregulation in the BM, which is essential for HSPC mobilisation, is affected by SNS inhibition and specifically abrogated after treatment with isoprenaline (isoproterenol), a non-selective β-adrenergic agonist. They further identified that this effect is dependent solely on the β_3_ adrenergic receptor, as treatment with BRL37344 (a β_3_-specific adrenergic agonist) is able to decrease SDF-1 production form MS-5 cells _in vitro_ and BM SDF-1 levels _in vivo_. Consistently the levels are increased when a specific antagonist is used. This β_3_-dependent SDF-1 decrease induced HSPC release _in vitro_ but did not cause mobilisation _in vivo_. The authors suggested that this discrepancy between the _in vitro_ and _in vivo_ observations could be due to a β-adrenergic-induced increase of HSPC homing and attachment, which cancelled the effect on mobilisation. For this purpose, further research was performed in specific animal models where vasculature-homing and adhesion were compromised. In these models, β_3_-adrenergic stimulation significantly enhanced HSPC circulation, demonstrating direct involvement of the receptor in this process. Mendez-Ferrer and colleagues
also attempted to determine the intermediate cell population that implements this effect and identified a group of adherent, SDF-1-producing stromal cells, distinct from the osteoblasts, which only express the $\beta_3$ receptor (Méndez-Ferrer et al., 2008). The same group later described the mesenchymal nestin$^+$ stem cell population, as the HSC retaining, SNS responsive cell type through which HSC homing and mobilisation are controlled (Méndez-Ferrer et al., 2010).

### 8.1.2.3 Additional neurotransmitter receptors with roles in haemopoiesis

A variety of other neurotransmitter receptors have also been detected on HSPC and some have been shown to mediate the function of these cells. Orexin receptors (1 and 2), for instance, are expressed in primitive CD34$^+$ cells, whilst they are absent in differentiated blood cells (Steidl et al., 2004). Stimulation of CD34$^+$ cells with orexins decreased intracellular cAMP, increased Ca$^{2+}$ release, decreased fibronectin adhesion and increased early progenitor clonogenicity (Bruns et al., 2008). Immature CD34$^+$ cells also express the neurokinin receptors NK-1 and NK-2 (Hiramoto et al., 1998), which bind to substance P. Exposure to substance P can increase proliferation of immature CB CD34$^+$ cells, *in vitro*. Neurokinin A, on the contrary, can suppress HSPC growth (Murthy et al., 2008). Interestingly, substance P production in the BM is directly affected by SDF-1 levels (Corcoran et al., 2007). This suggests that the circadian fluctuations of SDF-1 (Méndez-Ferrer et al., 2008) also mediate the substance P impact on CD34$^+$ cells in the BM.

Primitive HSPC also express the Calcitonin gene-related peptide (CGRP) receptor and stimulation of these cells with the CGRP, which is expressed in the BM (Mignini et al., 2003) can enhance clonogenicity (Harzenetetter et al., 2002). Similarly HSPC express opioid receptors (Williams et al., 2001;

Other neurotransmitter receptors expressed on HSPC include the adenosine receptor (A\(_2\)R) and the \(\gamma\)-aminobutyrate receptor, although their function has not been described so far (Steidl et al, 2004; Streitová et al, 2010). Finally, the neurotransmitters angiotensin, somatostatin, serotonin and corticotrophin-releasing hormone have been shown to affect HSC function, mainly by enhancing HSC expansion in culture (Oomen, 2000; Steidl et al, 2004; Peng et al, 2005; Ferone et al, 2006; Yang et al, 2007).

8.1.3 In vivo modulation of HSPC behaviour with neurotransmitters

Recent research, mainly by the laboratories of Frenette and Lapidot, has produced in vivo evidence of the direct and indirect effects of dopamine and adrenergic receptors on haemopoietic stem cell behaviour which have expanded our understanding of the interaction of the nervous system on HSPC (discussed in section 1.7.4).

The Lapidot group explored the effects of G-CSF exposure on the expression of neurotransmitter receptors D\(_3\), D\(_5\) and \(\beta_2\) on HSPC (Spiegel et al, 2007). They reported that MPB progenitors have higher expression of these three receptors in comparison to CB and BM. Exposure of CB CD34\(^+\) cells to G-CSF, however, increases expression of these neurotransmitter receptors. Furthermore, in vitro migration assays revealed that MPB and G-CSF stimulated CB progenitors (but not unstimulated CB cells) migrate towards dopamine and norepinephrine (Spiegel et al, 2007).
The authors also used the neurotransmitters epinephrine, norepinephrine and dopamine to modulate the \textit{in vitro} and \textit{in vivo} behaviour of haemopoietic progenitors. An earlier study had demonstrated that epinephrine and norepinephrine treatment of BM progenitors significantly increases their clonogenicity, although this effect is reversed at high doses (≥ 10^{-3} M) of the neurotransmitters (Fonseca \textit{et al}, 2005). Similarly, Spiegel and colleagues report an increase in clonogenicity of MPB and G-CSF activated CB cells after exposure to norepinephrine or dopamine agonists. This \textit{in vitro} observation was confirmed \textit{in vivo} where a daily administration of epinephrine, at 50µg/mouse/day, for 6 days, significantly increased the number of HSPC in the animal BM. This was abolished when a β_{2} antagonist was used. They hypothesised that this is related to Wnt pathway activation. Wnt signalling and β-catenin have well described roles in haemopoietic stem cell proliferation and self-renewal (Bhardwaj \textit{et al}, 2001; Reya \textit{et al}, 2003). Spiegel \textit{et al} show that both epinephrine and dopamine treatments induce an accumulation of β-catenin in the cells. The Wnt-pathway antagonist, Dickkopf-1 (Dkk-1), abrogated the effect these neurotransmitters had on HSPC clonogenicity (Spiegel \textit{et al}, 2007). Proliferation of HSPC in the BM after epinephrine administration could also be related to the increased production of IL-6 from supporting BM stromal cells, after exposure to the neurotransmitter (Fonseca \textit{et al}, 2005).

A daily administration of epinephrine (50µg/mouse/day, for 6 days) was also shown to increase progenitor mobilisation in the treated animals. This is in line with the increased random migration of epinephrine treated progenitors \textit{in vitro} (Spiegel \textit{et al}, 2007). In a different experimental setting, Lucas and colleagues demonstrated that increased concentration of norepinephrine in the BM (via inhibition of the re-uptake mechanism) also leads to increased G-CSF-induced HSPC mobilisation (Lucas \textit{et al}, 2012).
8.1.4 Increased HSC engraftment after neurotransmitter stimulation

One of the most remarkable observations made by Spiegel and co-workers was the effect of *ex vivo* neurotransmitter treatment on the engraftment of MPB and CB CD34⁺ cells. Dopamine agonist (SKF) treatment (1µM) increased engraftment of MPB progenitors by 2-fold. The same cells had 2.6-fold higher engraftment after norepinephrine treatment (1µM) and 3.4-fold higher engraftment after epinephrine treatment (10nM). Consistently, CB CD34⁺ cell engraftment was enhanced after G-CSF stimulation combined with neurotransmitter treatment. There was a 1.5-fold increase in CB CD34⁺ engraftment after dopamine agonist treatment (1µM) and that value increased by 3-fold for norepinephrine (1µM). Pre-treatment with epinephrine (10nM) combined with G-CSF, increased CB CD34⁺ cell engraftment by 9-fold, compared to G-CSF stimulation alone. In fact, epinephrine treatment increased CB progenitor engraftment by 30-fold when used at the higher concentration of 1µM (versus G-CSF alone), although high sample variability was observed at this concentration. Finally, in *in vivo* assays where the animals were injected with limiting numbers of CB progenitors, epinephrine (10nM) and norepinephrine (1µM) significantly increased engraftment levels. Epinephrine treatment in particular, increased engraftment to levels higher than those achieved with double the number of cells pre-treated with G-CSF alone. No effects on HSC differentiation from the neurotransmitter treatments was observed in these experiments.

The authors confirm the Wnt pathway involvement in this phenomenon by demonstrating that Wnt inhibition reverses the positive effect of norepinephrine treatment on CB CD34⁺ cell engraftment (Spiegel *et al*, 2007). Further expanding on these observations Aicher and colleagues showed that Dkk-1 controls the endosteal environment and can induce the release of the major bone-resorbing protease cathepsin K, which leads to HSPC
mobilisation (Aicher et al, 2008). Wnt signaling was shown to be significantly downregulated in primitive HSC, but induced expression of β-catenin can expand these cells and restore the multipotency in committed progenitors (Reya et al, 2003; Baba et al, 2005, 2006). In vivo, enforced Wnt stimulation has been shown to lead to haemopoietic failure due to deficient HSC differentiation (Scheller et al, 2006; Kirstetter et al, 2006). Inhibition of Wnt signaling on HSPC with Dkk-1 lead to increased cycling, and reduced haemopoiesis post transplantation (Fleming et al, 2008).

Overall, research so far has demonstrated a direct and indirect control of haemopoietic stem cell proliferation, migration, engraftment and mobilisation via neurotransmitter signaling. SNS hormones released in the BM act on β3+ stromal cells, reducing SDF-1 production and inducing HSPC release. They also have an effect on β2 expressing osteoblasts, promoting bone remodelling, which alters the niche microenvironment and promotes mobilisation. Finally, they directly control HSC which express dopamine and adrenergic receptors and can modulate their proliferation, migration and engraftment. So far, however, no data has been published on the effect of cytokine exposure on the expression and function of neurotransmitter receptors on CD34+ cells.

8.1.5 Chapter aims

The aim of this part of the work was to explore the expression of neurotransmitter receptors (β2, D3 and D5) on MPB HSPC and to study their function and co-expression with other important receptors as well as the impact of cytokine culture.

The potential impact of direct neurotransmitter stimulation on in vivo functions of transplanted HSPC, as suggested by Spiegel et al, is relevant to the subject of this thesis, as it could be utilised to correct the cytokine-induced engraftment defect of ex vivo cultured HSPC. Hence, the epinephrine
stimulation protocol was optimised and its effects on the *in vitro* and *in vivo* properties of fresh and cultured HSPC were investigated.

### 8.2 Results

#### 8.2.1 Neurotransmitter receptor expression

**8.2.1.1 Cytokine culture effect on neurotransmitter receptor expression**

Adrenergic and dopamine receptor expression by MPB cells has been previously reported on a small number of donors (Spiegel *et al.*, 2007). Expression of β2, D3 and D5 was initially confirmed by FACS in 10 MPB samples. As shown in Figure 8.1 A β2 adrenergic receptor is expressed in a subpopulation of CD34+ cells from MPB samples, with a mean expression level of 13.30 ± 1.955% (Figure 8.1 B). D3 and D5 receptors, however, had a more homogenous pattern of expression, as 82.81 ± 7.647% and 89.98 ± 5.587% of CD34+ cells expressed the two receptors, respectively (Figure 8.1). Expression levels for the three receptors were compared between freshly isolated and thawed MPB CD34+ cells. The freeze-thawing process was found to mildly decrease expression of D3 and D5 but the difference was not statistically significant. The majority of the experiments described below were performed using thawed cells.

Culture of hCD34+ cells with cytokines alters the function and expression profile of these cells in several ways (discussed in Chapters 3 and 6). Here the effect of culture in cytokines on the expression of neurotransmitter receptors β2, D3 and D5 was tested. After 72 hours of culture in cytokines the percentage of β2 expressing cells increased by 1.3-fold (p<0.01, paired Student’s t-test) (Figure 8.2 A). The protein expression of β2 per cell (as indicated by MFI ratio) also increased in a similar manner (1.5-fold) and the effect was statistically significant (p<0.05, paired Student’s t-test) (Figure 8.2 B). D3 and D5 expression, however, decreased after cytokine activation. The
Figure 8.1: $\beta_2$, $D_3$, and $D_5$ expression in mobilised PB CD34$^+$ cells. A: Representative histograms of $\beta_2$, $D_3$, and $D_5$ expression on fresh mobilised PB CD34$^+$ cells, as analysed by FACS. Isotype controls are indicated with the dashed line. B: Percentage of cells expressing $\beta_2$, $D_3$, and $D_5$ in fresh CD34$^+$ cells (FACS analysis). Levels shown for each donor with medians indicated.
Figure 8.2: $\beta_2$, $D_3$ and $D_5$ expression in fresh and cytokine cultured hCD34+ cells. A: Percentage of fresh and cytokine cultured (3 days) CD34+ cells positive for $\beta_2$, $D_3$ and $D_5$ as analysed by FACS. B: Fold difference in Mean Fluorescence Intensity (MFI), compared to controls stained with isotype-matched antibodies, at 0 and 72 hours post cytokine activation. Levels shown for each donor with pre and post samples of the same donor connected. *p<0.05, **p<0.01, ***p<0.001, paired Student’s t-test.
percentage of cells positive for the D\textsubscript{3} receptor decreased by 68% and by 55% for D\textsubscript{5} after 72 hours of cytokine culture (Figure 8.2 A). The MFI ratio for both receptors also dramatically decreased with culture. This outcome was particularly pronounced for D\textsubscript{5} which had a higher MFI ratio on fresh cells. All differences described here were statistically significant (p<0.05). CD34 expression, which was used as a control, did not change significantly in these experiments after 72 hours of culture in cytokines (Figure 8.2 A).

Timecourse analysis of the expression of the three receptors was performed next, to determine the kinetics of the changes described above. B\textsubscript{2} expression increased gradually reaching maximum levels at 48 hours. The MFI ratio for B\textsubscript{2} rapidly increased after 16 hours in culture (Figure 8.3). In contrast, D\textsubscript{3} and D\textsubscript{5} expression gradually decreased over the 72 hours of cytokine culture with the MFI ratio dropping more rapidly, in the first 24 hours (Figures 8.4 and 8.5).

8.2.1.2 Co-expression with CXCR4 and CD26

CXCR4 and CD26 expression has also been shown to increase with exposure to cytokines (Chapters 3 and 7). B\textsubscript{2} positive cells are a subgroup of the CD34\textsuperscript{+} cell population which increases with cytokine activation. The aim of the next experiments was to further characterise this subgroup by testing for co-expression of the receptors CXCR4 and CD26 which play significant roles in HSPC homing and engraftment. Fresh and cultured CD34\textsuperscript{+} cells were co-stained for CXCR4 and B\textsubscript{2} or CD26 and B\textsubscript{2} expression. Co-expression of the pairs of receptors was quantified by FACS analysis, as shown in a representative plot, for CXCR4 and B\textsubscript{2} expression (Figure 8.6 A). As seen in Figure 8.6 B (top panel) in fresh cells 44.55% of the CD34\textsuperscript{+}/CXCR4\textsuperscript{+} cells also expressed the B\textsubscript{2} receptor. After culture in cytokines, however, the majority of the CD34\textsuperscript{+}/CXCR4\textsuperscript{+} cells (77.97%) also acquired expression of B\textsubscript{2}. Similarly, only 56.14% of the CD34\textsuperscript{+}/CD26\textsuperscript{+} cells co-expressed B\textsubscript{2} but this
Figure 8.3: Timecourse of changes in β_2 expression with cytokine activation. Top panel: Percentage of hCD34^+ cells positive for β_2 at 0, 16, 24, 48 and 72 hours post cytokine activation. Bottom panel: Fold difference in Mean Fluorescence Intensity (MFI), compared to control stained with isotype-matched antibody, at 0, 16, 24, 48 and 72 hours post cytokine activation. Mean ± SEM, n=3.
Figure 8.4: Timecourse of changes in D₃ expression with cytokine activation. Top panel: Percentage of hCD34⁺ cells positive for D₃ at 0, 16, 24, 48 and 72 hours post cytokine activation. Bottom panel: Fold difference in Mean Fluorescence Intensity (MFI), compared to control stained with isotype-matched antibody, at 0, 16, 24, 48 and 72 hours post cytokine activation. Mean ± SEM, n=3.
Figure 8.5: Timecourse of changes in D₅ expression with cytokine activation. Top panel: Percentage of hCD34⁺ cells positive for D₅ at 0, 16, 24, 48 and 72 hours post cytokine activation. Bottom panel: Fold difference in Mean Fluorescence Intensity (MFI), compared to control stained with isotype-matched antibody, at 0, 16, 24, 48 and 72 hours post cytokine activation. Mean ± SEM, n=3.
Figure 8.6: Co-expression of $\beta_2$ with CXCR4 and CD26 on HSPC. A: Representative FACS plots of fresh and cultured CD34$^+$ cells stained for CXCR4 and $\beta_2$ expression. B, top panel: Percentage of CXCR4$^+$ cells which co-express $\beta_2$ before and after cytokine culture. B, bottom panel: Percentage of CD26$^+$ cells which co-express $\beta_2$ before and after cytokine culture. Mean ± SEM, n=3.
value increased to 94.70% after culture in cytokines (Figure 8.6 B, bottom panel). These results indicate that the co-expression of CXCR4 and CD26 with $\beta_2$ increases after culture in cytokines.

The reverse was observed for the co-expression of these two receptors with neurotransmitter receptors D$_3$ and D$_5$. In fresh cells 71.14% of the CD34$^+$ cells expressing CXCR4 were also positive for D$_3$ whereas after culture in cytokines this percentage was reduced to 32.09% (top panel Figure 8.7). Similarly, while an average of 63% of fresh CD26 expressing cells also expressed D$_3$, this was reduced to 41.02% after culture (bottom panel Figure 8.7). Moreover, before culture 85.93% of the CXCR4$^+$ cells also expressed D$_5$, whereas after cytokine culture this was reduced to 47.88% (Figure 8.8, top panel). CD26 expression coincided with D$_5$ expression for 92.16% (of the CD26 expressing fresh cells) but was decreased to 40.69% after culture (Figure 8.8, bottom panel). These observations collectively show that after culture in cytokines, while the percentage of CXCR4 and CD26 expression increases, dopamine receptor expression within these populations is not similarly altered, leading to decreased co-expression levels which may impact on the SNS-driven trafficking and attachment of the expanded cells.

**8.2.2 Clonogenicity of sub-populations**

The effect of SNS signaling in HSPC proliferation and colony formation has been partly investigated by two studies which demonstrated an increase in HSPC clonogenicity after epinephrine, norepinephrine and dopamine treatment (Fonseca et al, 2005; Spiegel et al, 2007). It can be assumed that this is related to the $\beta_2$, D$_3$ and D$_5$ expression on these cells. The aim of the next experiments was to study the clonogenicity of CD34$^+$ cells in relation to expression of these receptors. Furthermore the effect of HSPC culture in this context was investigated to reveal any cytokine-induced changes in the clonogenicity of the different neuroreceptor expressing subpopulations.
Figure 8.7: Co-expression of D$_3$ with CXCR4 and CD26 on HSPC. Fresh and cultured CD34$^+$ cells were co-stained for CXCR4 and D$_3$ or CD26 and D$_3$. Top panel: Percentage of CXCR4$^+$ cells which co-express D$_3$ before and after cytokine culture. Bottom panel: Percentage of CD26$^+$ cells which co-express D$_3$ before and after cytokine culture. Mean ± SEM, n=3.
Figure 8.8: Co-expression of D₅ with CXCR4 and CD26 on HSPC. Fresh and cultured CD34⁺ cells were co-stained for CXCR4 and D₅ or CD26 and D₅. Top panel: Percentage of CXCR4⁺ cells which co-express D₅ before and after cytokine culture. Bottom panel: Percentage of CD26⁺ cells which co-express D₅ before and after cytokine culture. Mean ± SEM, n=3.
To do this analysis, fresh and cytokine cultured cells were stained with the β₂, D₃ and D₅ antibodies and a secondary FITC conjugated antibody. The samples were analysed by FACS and six gates were set up according to FITC expression starting from the negative population and covering up to the highest expressing cells (Figure 8.9, top panel). Cells were sorted from each of these gates directly into methylcellulose medium in a 96 well plate. Ten cells from each gate were sorted in each well of the plate, ten times for each gate (Figure 8.9, bottom panel). Appropriate controls were sorted in methylcellulose to confirm that the staining process and antibody binding did not affect clonogenicity of the samples. Colonies were scored 14 days later and the results are presented separately for total clonogenicity as well as the BFU-E and CFU-GM production for each expression subgroup.

The levels of β₂ expression did not have any significant impact on the clonogenicity of fresh cells (Figure 8.10). Total clonogenicity and CFU-GM production appeared to be higher in cells expressing high levels of β₂ but these differences were not statistically significant. In cytokine cultured cells, however, this changed, as both BFU-E and CFU-GM colony production (and subsequently total clonogenicity) decreased as expression of β₂ increased. More specifically, the cells that had no expression of β₂ had mean clonogenic frequency of 3.183 ± 0.5527/10 cells whereas the high expressors produced 0.1199 ± 0.0705 colonies/10 cells (p<0.05, paired Student’s t-test). The differences between negative and high expressing cells in terms of clonogenicity were also significant when BFU-E and CFU-GM were analysed separately (p<0.05, paired Student’s t-test).

Similarly, D₃ expression was not associated with the clonogenic potential of fresh cells (Figure 8.11). BFU-E production appeared to decline as D₃ expression increased but this was not statistically significant, and total clonogenicity did not vary with D₃ expression levels. In cytokine activated
Figure 8.9: Clonogenicity in relation to receptor expression. Fresh and cultured hCD34+ cells were analysed by FACS and six gates were set up separating different expression levels (as indicated in a representative example on the top panel). Ten cells/well from each gate were then sorted in a 96-well plate well, directly into methylcellulose. Ten wells were seeded for each gate, as shown in the bottom panel. Colonies for each expression group were scored 14 days later.
Figure 8.10: Clonogenicity of CD34+ cells with different levels of β2 expression. Fresh (clear) and cultured (grey) hCD34+ cells were FACS sorted into methylcellulose depending on their β2 expression profile. Colonies were scored 14 days later and the values are presented separately for CFU-GM, BFU-E and total colony production (as indicated). Mean ± SEM, *p<0.05, paired Student’s t-test, n=3.
Figure 8.11: Clonogenicity of CD34⁺ cells with different levels of D₃ expression. Fresh (clear) and cultured (grey) hCD34⁺ cells were FACS sorted into methylcellulose depending on their D₃ expression profile. Colonies were scored 14 days later and the values are presented separately for CFU-GM, BFU-E and total colony production (as indicated). Mean ± SEM, *p<0.05, **p<0.01, n=3.
cells, however, both CFU-GM and BFU-E production declined as $D_3$ expression increased and subsequently total clonogenicity was significantly reduced ($p<0.01$, paired Student’s t-test).

The same observations are shown in Figure 8.12 for $D_5$ expression. In fresh cells, total clonogenicity did not follow an expression-related pattern. The higher $D_5$ expressors appeared to have lower BFU-E and higher CFU-GM production but these differences were not statistically significant. On the other hand, after culture in cytokines, higher $D_5$ expression was associated with decreased clonogenicity (both myeloid and erythroid). The lowest expressors, for example, had a frequency of $2.354 \pm 0.4288$ colonies/10 cells whereas the cells expressing the highest levels of $D_5$ produced $0.7670 \pm 0.3989$ colonies/10 cells ($p<0.05$, paired Student’s t-test). The reduction for BFU-E and CFU-GM production with increased $D_5$ expression was also significant ($p<0.05$, paired Student’s t-test).

Overall cytokine culture appears to reduce the clonogenic potential of the neurotransmitter receptor expressing cells.

**8.2.3 Epinephrine treatment**

Treatment of MPB and G-CSF stimulated CB progenitors with the neurotransmitters dopamine, epinephrine and norepinephrine was shown to increase engraftment in immunodeficient animals. Amongst the three neurotransmitters, epinephrine was shown to have the most pronounced effects in both CB and MPB engraftment, reaching 3.4-fold of control engraftment for MPB and a maximum 30-fold increase for CB HSPC (Spiegel et al., 2007). For this reason, epinephrine was selected for the next series of experiments, to attempt to modulate the behaviour of cytokine cultured MPB CD34$^+$ cells. Initially, the effect of culturing the haemopoietic progenitors in
Figure 8.12: Clonogenicity of CD34+ cells with different levels of D5 expression. Fresh (clear) and cultured (grey) hCD34+ cells were FACS sorted into methylcellulose depending on their D5 expression profile. Colonies were scored 14 days later and the values are presented separately for CFU-GM, BFU-E and total colony production (as indicated). Mean ± SEM, *p<0.05, n=3.
the presence of epinephrine was assessed with expression analysis and \textit{in vitro} functional assays.

\subsection*{8.2.3.1 Expression of surface receptors}

Fresh cells were stained for $\beta_2$, $D_3$, $D_5$ and CD34/CD45 expression and analysed by FACS. Cells from each donor were also cultured in the standard cytokine mix with or without the addition of epinephrine at 10nM. After 3 days these cells were stained for the same receptors. Figure 8.13 confirms the cytokine effects on $\beta_2$, $D_3$ and $D_5$ expression as discussed in section 8.2.1.1. The inclusion of epinephrine in culture had no significant effect on the expression of these receptors.

Expression of CD26 and CXCR4 was similarly unaffected by epinephrine treatment. As shown in Figure 8.14 culture in epinephrine did not alter the expression of either of these receptors. Epinephrine stimulation had no effect on the expression of several other relevant adhesion and homing receptors (CD44, CD49d, CD49e, N-cadherin and L-selectin) (Figure 8.14).

Moreover the co-expression of CXCR4 and CD26 with neurotransmitter receptors ($\beta_2$, $D_3$ and $D_5$) is similarly unaffected by the epinephrine stimulation (Figure 8.15).

\subsection*{8.2.3.2 Clonogenicity}

Adrenergic hormones have been previously reported to aid proliferation and clonogenicity of HSPC from BM, CB and MPB (Fonseca \textit{et al}, 2005; Spiegel \textit{et al}, 2007). In Figure 8.16, the clonogenicity of fresh and cultured MPB CD34$^+$ cells was compared, with and without epinephrine treatment. Mean CFU-GM production increased with culture in epinephrine from 227.3 $\pm$ 12.51 to 285.1 $\pm$ 23 colonies per 1000 cells. Similarly BFU-E numbers increased from 94.36 $\pm$ 10.38 to 116.9 $\pm$ 10.18, after culture in epinephrine and
Figure 8.13: Effect of epinephrine treatment on $\beta_2$, $D_3$, $D_5$ and CD45/CD34 expression:
Fresh and 72 hours cultured hCD34$^+$ cells, with or without epinephrine pre-treatment, were stained for $\beta_2$, $D_3$, $D_5$ and CD34/CD45 expression and analysed by FACS. Levels shown separately for each donor. Differences between “cultured” and “cultured+ Epi” cells are not statistically significant for all the graphs, as analysed by Student’s paired t-test.
Figure 8.14: Effect of epinephrine treatment on receptor expression. Cultured hCD34+ cells, with or without epinephrine pre-treatment, were stained for CD44, CD49d, CD49e, N-cadherin, L-selectin, CXCR4 and CD26 expression and analysed by FACS. Mean ± SEM shown, n=4.
Figure 8.15: Effect of epinephrine on co-expression of $\beta_2$, $D_3$ and $D_5$ with CXCR4 and CD26 on cultured CD34$^+$ cells. Cultured CD34$^+$ cells were co-stained with CXCR4 or CD26 with the neurotransmitter receptors $\beta_2$, $D_3$ and $D_5$. Co-expression was analysed as described in section 8.2.1.2 and shown in Figure 8.6. Top panel: Percentage of CXCR4$^+$ cells which co-express $\beta_2$, $D_3$ and $D_5$ when cultured with or without epinephrine. Bottom panel: Percentage of CD26$^+$ cells which co-express $\beta_2$, $D_3$ and $D_5$ when cultured with and without epinephrine. Mean ± SEM, n=3.
Figure 8.16: Effect of epinephrine treatment on clonogenicity. 1000 cultured hCD34+ cells, with or without epinephrine treatment, were seeded in methylcellulose. Colonies were scored 14 days later and the values are presented separately for CFU-GM, BFU-E and total colony production (as indicated). The clonogenic frequency of each donor is shown separately. The differences between the two groups were analysed by paired Student’s t-test, p<0.01 in all cases.
subsequently total clonogenicity also increased after culture in epinephrine (from 318.6 to 399.6 colonies/1000 cells). The effect size of this treatment is small but statistically significant (p<0.01 for all three groups, as analysed by paired Student’s t-test).

8.2.3.3 Migration

Epinephrine administration in vivo has been shown to increase random migration of BM mononuclear cells, in vitro. Moreover, MPB and G-CSF stimulated CB progenitors have shown chemotaxis towards dopamine and norepinephrine (Spiegel et al, 2007). No data on HSPC chemotaxis towards an epinephrine gradient have been reported. Moreover, the effect of epinephrine treatment on fresh and cultured MPB hCD34+ cell migration has not been studied. This section aims to address these questions.

Fresh MPB CD34+ cells were used in migration assays towards an SDF-1 (100ng/ml) or epinephrine (1µM) gradient. A combination of the two proteins was also used to assess any synergistic effects and random migration towards medium was used as a control. CFC migration was determined with colony assays, by seeding a proportion of the migrated cells in methylcellulose, as described in section 2.2.2. The same protocol was used with cells cultured in the standard cytokine mix with or without epinephrine for 3 days.

As previously discussed (section 3.2.6), fresh hCD34+ cells had low random migration (3.343 ± 1.217%) but they responded to an SDF-1 gradient reaching migration levels of 6.430 ± 1.410%. Interestingly, fresh CD34+ cells had some chemotaxis towards epinephrine (5.388 ± 1.121%) but this was not statistically different from baseline levels. Combination of the two proteins (SDF-1 and epinephrine) further increased migration to 8.508 ± 2.521% which was significantly higher than baseline but not compared to SDF-1 or epinephrine alone (Figure 8.17).
Figure 8.17: Effect of epinephrine on HSPC migration. Fresh and 72 hours cultured hCD34\(^+\) cells, with or without epinephrine (10nM), were used in in vitro migration assays. Migration towards medium or a gradient of SDF-1 (100ng/ml), epinephrine (1\(\mu\)M) or the combination of the two proteins (at the aforementioned concentrations) was tested. A proportion of the migrated cells was seeded in methylcellulose (with appropriate controls) to measure CFC migration levels. Mean ± SEM is shown, n=3 (duplicates were performed for each donor). \(*p<0.05, **p<0.01, ***p<0.001,\) Student's t-test.
Cytokine activated cells had increased random migration (10.96 ± 1.518%) and responded to an SDF-1 gradient reaching significantly higher levels of migration (22.83 ± 2.934%). In response to epinephrine, cytokine cultured CD34<sup>+</sup> cells migrated at levels of 16.37 ± 1.581%, which was significantly higher than baseline migration. This is consistent with the increased β<sub>2</sub> expression detected on cultured cells (section 8.2.1). A combination of both proteins did not significantly increase migration further than the levels achieved towards SDF-1 alone (25.07 ± 2.982%) (Figure 8.17).

Culture of CD34<sup>+</sup> cells in cytokines with epinephrine (10nM) did not significantly alter random migration (14.36 ± 2.423%). Migration towards an SDF-1 gradient was similar to the levels achieved without the epinephrine treatment (25.12 ± 2.976%). The epinephrine treated cells, however, did not specifically migrate towards epinephrine. The migration levels achieved were similar to baseline migration (10.83 ± 1.628%). This may be because previous culture in epinephrine desensitised the cells to the neurotransmitter. Finally, combination of the two proteins achieved migration levels similar to those achieved by SDF-1 alone (25.58 ± 2.253%) (Figure 8.17).

8.2.3.4 Adhesion

8.2.3.4.1 Epinephrine titration

Next, the effect of epinephrine treatment on adhesion was assessed. CD34<sup>+</sup> cells were cultured in the standard cytokine mix, with increasing concentrations of epinephrine, over 72 hours. The cells were then used in adhesion assays to N-cadherin (10µg/ml) as previously described, and specific progenitor adhesion was measured (Figure 8.18). Epinephrine was found to have a positive effect on the adhesion of cytokine cultured CD34<sup>+</sup> cells to N-cadherin, at a concentration of 5nM (24.5 ± 2.111% for untreated vs. 34.67 ± 5.390% for 5nM epinephrine treated cells). At higher
Figure 8.18: Epinephrine titration on adhesion to N-cadherin. CD34+ cells were cultured with cytokines and epinephrine (at the concentrations indicated) for 72 hours. The cells were then used in adhesion assays on N-cadherin (10 µg/ml) and progenitor adhesion was measured with colony assays. Mean ± SEM is shown, *p<0.05, n=3. Analysis by Student's t-test.
concentrations culture of CD34\(^+\) cells with epinephrine was found to significantly decrease adhesion to epinephrine reaching \(7.450 \pm 1.150\%\) at 100nM epinephrine.

Using the optimal epinephrine concentration of 5nM this protocol was repeated with more donors and compared to the adhesion achieved by freshly isolated CD34\(^+\) cells. Cytokine activation significantly reduced N-cadherin adhesion (as discussed in section 6.2.2). Fresh cells had \(36.20 \pm 2.656\%\) specific adhesion to N-cadherin whereas after 3 days in cytokines that value was reduced to \(16.20 \pm 2.656\%\). When the cytokine culture, however, took place in the presence of epinephrine (5nM) for 72 hours, the levels of adhesion increased significantly reaching those achieved by fresh cells (\(42.45 \pm 4.189\%\)) (Figure 8.19).

### 8.2.3.4.2 Protocol optimisation in adhesion

To better describe the effect of epinephrine pre-treatment, different incubation times were tested next. Epinephrine was added in culture 72 or 24 hours before the cells were used or was only present for the duration of the assay (90 minutes). These three conditions were compared to untreated cytokine cultured cells in further adhesion assays to N-cadherin (Figure 8.20). Addition of epinephrine (5nM) for the duration of the assay had a small but significant effect \((p<0.05)\) on the resulting adhesion to epinephrine, increasing adhesion from \(17.55 \pm 2.126\%\) to \(26.91 \pm 3.584\%\). When epinephrine was added for the last 24 hours of culture, before the cells were used for the assay, the levels of adhesion to N-cadherin were further increased to \(46.29 \pm 12.59\%\) \((p<0.01\) compared to control cells not exposed to epinephrine). Finally the 72 hour culture in epinephrine with cytokines increased adhesion to \(36.08 \pm 4.808\%\) \((p<0.01\) compared to control cells, not exposed to epinephrine).
Figure 8.19: Effect of epinephrine on adhesion to N-cadherin. Fresh and cytokine cultured hCD34+ cells with or without epinephrine (5nM, 72 hours) were used in in vitro adhesion assays to N-cadherin. Progenitor adhesion was measured with colony assays. Mean ± SEM is shown, n=6 (triplicates were performed for each donor). **p<0.01, ***p<0.001, Student's t-test.
Figure 8.20: Epinephrine treatment optimisation on adhesion to N-cadherin. CD34+ cells were cultured with cytokines and used in adhesion assays to N-cadherin (10µg/ml) either untreated or co-cultured with epinephrine (5nM) for 72 or 24 hours before use or with epinephrine (5nM) added only for the duration of the assay. Progenitor adhesion was measured with colony assays. Mean ± SEM is shown, n=4. *p<0.05, **p<0.01, analysis by Student's t-test.
Based on these results, overnight epinephrine stimulation at a concentration of 5nM was selected as the optimal protocol to improve HSPC adhesion. Next, the effect of this protocol on progenitor adhesion to VCAM-1 and osteopontin was also tested (Figure 8.21). Adhesion of HSPC to N-cadherin, osteopontin and VCAM-1 was previously shown to be significantly reduced after cytokine culture (section 6.2.2). Using this protocol of overnight stimulation with epinephrine, however, this cytokine-induced defect in adhesion is reversed for N-cadherin and osteopontin, but not for VCAM-1. N-cadherin adhesion reached 37.30 ± 1.505% (mean ± SEM) for fresh HSPC but was reduced to 20.61 ± 1.674% after culture in cytokines. Epinephrine stimulation increased the N-cadherin adhesion of cytokine cultured HSPC to 38.49 ± 3.091%. Similarly, osteopontin adhesion decreased from 27.12 ± 2.554% to 4.449 ± 0.9084% after culture in cytokines. After overnight pre-treatment with epinephrine, however, the osteopontin adhesion of cultured HSPC increased to levels similar to those of fresh cells (24.94 ± 3.208%). Cytokine culture decreased VCAM-1 adhesion from 66.12 ± 4.921% to 52.10 ± 4.031%. Overnight stimulation did not change VCAM-1 adhesion levels after culture (52.80 ± 4.832%).

Despite the effect of epinephrine stimulation on adhesion, the expression of the relevant receptors (N-cadherin, osteopontin and VLA-4) was not altered when the cells were pre-stimulated with epinephrine (Figure 8.14).

8.2.3.5 Ex vivo model

As epinephrine stimulation for 24 hours prior to use was found to increase adhesion to N-cadherin and osteopontin in vitro, we used the same protocol to pre-treat CD34+ cells that were then used in the ex vivo attachment model previously described (Chapter 5). Fresh and cultured cells either with or
Figure 8.21: Effect of epinephrine on adhesion to N-cadherin, osteopontin and VCAM-1.

CD34+ cells were cultured with cytokines with or without a 24 hour epinephrine stimulation prior to use. The cells were then used in adhesion assays to N-cadherin (10µg/ml), osteopontin (5µg/ml) and VCAM-1 (10µg/ml). Specific progenitor adhesion was measured with colony assays. Mean ± SEM is shown, n=3 (triplicates were performed for each donor). ***p<0.0001, *p<0.05, analysis by Student’s t-test.
without a 24 hour pre-incubation with epinephrine (at 5nM) were infused in rat BM cavities *ex vivo*. Progenitor attachment was measured with colony assays. As previously shown (section 5.3.3), cytokine culture significantly decreased HSPC attachment. Fresh hCD34⁺ cells had 881.9 (489.9-1514) attached CFC whereas after culture this decreased to 503.3 (262.9-972.8, p<0.05, Mann-Whitney test). Epinephrine stimulation of cultured cells increased progenitor attachment to 691 (450.8-1636) but the difference compared to untreated cells did not reach significance in this series of experiments (Figure 8.22).

8.2.3.6 Homing

Epinephrine stimulation has been shown to increase engraftment of fresh MPB CD34⁺ cells, suggesting that arrival of the HSPC in the BM cavities happens in a timely fashion. On the other hand, BM mononuclear cells have increased random migration after *in vivo* epinephrine administration which could translate into increased localisation of the infused HSPC in non-haemopoietic tissue, *in vivo* (Spiegel *et al*, 2007). The effect of epinephrine treatment on the short-term homing of HSPC has not been directly studied. Here, epinephrine treatment (72 hour stimulation with 5nM epinephrine prior to use) was used on cytokine cultured MPB hCD34⁺ cells. The cells were then infused in pre-irradiated, β₂m/NOD/SCID mice, to test progenitor short-term homing (as described in section 2.3.4.1).

Pre-treatment of cultured HSPC with epinephrine (for 72 hours) significantly increased the levels of homing to the BM of the recipients. CD34⁺ cells that had been cultured in cytokines for 3 days, in the absence of epinephrine achieved median levels of homing 0.7971% (0.6627%-1.134%) whereas in the presence of epinephrine short-term homing increased to 1.868% (1.402%-2.270%, p<0.001, Mann-Whitney test) (Figure 8.23).
Figure 8.22: Epinephrine effect on HSPC attachment. CD34+ cells were cultured with cytokines with or without a 24 hour epinephrine stimulation prior to use. The cells were infused in the rat bones at a cell dose of $0.5 \times 10^6$/ml. Progenitor attachment was measured with clonogenic assays. Values for individual bones are shown with medians indicated. *p<0.5, Mann-Whitney test.
Figure 8.23: Epinephrine effect on HSPC homing. CD34+ cells were cultured with cytokines with or without a 72 hour epinephrine stimulation prior to use. The cells were infused IV (tail vein) into sublethally irradiated β2m/NOD/SCID mice at a dose of $2 \times 10^6$ cells per animal. Short-term homing is determined by the percentage of human CFC in the BM of the recipient animals, 20 hours after infusion. The levels of homing are shown separately for each animal. Statistical analysis by Mann-Whitney test, $p<0.001$. 
A number of receptors have been associated with HSPC homing. Among them, CD44 (Khaldoyanidi et al., 1996; Vermeulen et al., 1998; Pilarski et al., 1999), VLA-4 (Papayannopoulou et al., 1995; Frenette et al., 1998; Mazo et al., 1998; Papayannopoulou et al., 2001), VLA-5 (Peled et al., 2000) and L-selectin (Dercksen et al., 1995; Pratt et al., 2001) have been described to play important roles in this process. Epinephrine stimulation of cytokine cultured HSPC was found to have no effect on the percentage of positive cells in the population or the protein expression per cell (MFI ratio) for these homing receptors (Figure 8.14).

8.2.3.7 Engraftment

Next, we tested the effect of epinephrine treatment on the long-term engraftment of cultured MPB hCD34+ cells. Cultured cells with or without the epinephrine treatment (3 days, 5nM) were infused IV to pre-irradiated β2m/NOD/SCID mice (2x10^6 cells/animal). Long-term engraftment was assessed 8 weeks later by FACS analysis of the BM of the animals. For these experiments hCD34+ cells form two donors were injected to male and female mice (all aged 8 weeks at the time of injection).

Both the donors as well as the sex of the animals had an impact on the levels of engraftment achieved causing a significant baseline drift (discussed in section 2.3.4.2.3). Despite this, the epinephrine treatment increased median engraftment for each group (Figure 8.24). In order to summarise the data collected from these experiments, engraftment was expressed as fold increase in comparison to the average untreated engraftment levels for each donor and sex. This normalisation revealed a significant impact of epinephrine stimulation on engraftment of cytokine cultured cells (Figure 8.25). Epinephrine treated, cultured cells had 1.822-fold higher engraftment levels compared to their untreated counterparts (p<0.001, Mann-Whitney test).
Figure 8.24: Variation in engraftment with epinephrine treatment. CD34+ cells were cultured in cytokines, with or without a 72 hour epinephrine stimulation prior to use. The cells were infused IV into sublethally irradiated \(\beta_{2m}\)NOD/SCID mice at a dose of \(2 \times 10^6\) cells per animal. Engraftment was analysed 8-10 weeks later. The donors used as well as the sex of the animals were found to have a significant effect on the levels of engraftment achieved and are, therefore, shown separately. Females are shown in open circles and males in closed squares. Total engraftment for both donors used is shown in the top panels. Engraftment in female animals is shown in the middle panels and in male animals in the bottom panels.
Figure 8.25: Epinephrine effect on HSPC engraftment. CD34+ cells were cultured with cytokines with or without a 72 hour epinephrine stimulation prior to use. The cells were infused IV into sublethally irradiated β2m/NOD/SCID mice at a dose of 2x10^6 cells per animal. Engraftment was analysed 8-10 weeks later by assessing the percentage of hCD45+/hCD33+ or hCD45+/hCD19+ cells in the BM of the recipient animals by FACS. The levels of engraftment were normalised to the sex of the animals and the donor used and are expressed as fold increase in comparison to average untreated cell-engraftment. Statistical analysis by Mann-Whitney test, p<0.001.
Pre-treatment with epinephrine had no effect on the differentiation of the engrafted cells as compared with the cultured untreated cells (Figure 8.26), in agreement with observations made by Spiegel et al.

### 8.3 Discussion

The involvement of SNS in haemopoiesis is a concept which was proposed in the last 20 years and increasing evidence demonstrates a multifactorial impact which involves the supporting cells of the niches (stromal cells, osteoblasts and MSC) as wells as the HSPC themselves. The HSPC in particular, have been shown to express a variety of receptors that respond to signals from the nervous system. A recent study demonstrated expression of the dopamine receptors D$_3$ and D$_5$ as well as the adrenergic receptor β$_2$ on human CB, G-CSF MPB and BM progenitors. Here, high expression of D$_3$ and D$_5$ on MPB CD34$^+$ cells was confirmed as well as expression of β$_2$ on a subpopulation of HSPC (11.66%).

Exposure of G-CSF MPB CD34$^+$ cells to the standard cytokine mix reduced D$_3$ and D$_5$ expression, whereas β$_2$ expression was increased. These changes occurred gradually over the 72 hours of culture, although significant downregulation for both D$_3$ and D$_5$ occurs in the first 24 hours, which implies that these changes are not related to stem cell dilution with expansion. D$_3$ and D$_5$ downregulation would render the HSPC less sensitive to dopamine signals within the BM which is perhaps related to their reduced retention in this space post cytokine activation. Fresh, unmanipulated HSPC have high D$_3$ and D$_5$ expression, while β$_2$ expression is comparatively low. Thus, it can be assumed that an overexpression of β$_2$ is not necessary for the attachment and engraftment of HSPC in the BM space. On the contrary, as was shown on Figure 8.17 increased β$_2$ expression makes the activated HSPC more migratory (in vitro). This may lead them non-haemopoietic, epinephrine rich gradients in vivo. Overall, the effect of cytokine activation on the expression
Figure 8.26: Differentiation of engrafted cells with or without the epinephrine stimulation. Cultured hCD34+ cells were stimulated with epinephrine (or PBS) and then infused IV to sublethally irradiated β2M/NOD/SCID mice, at a cell dose of 2x10^6/animal. Engraftment was assessed by FACS analysis of bone marrow at 8-10 weeks. HCD19PE and hCD33APC antibodies were used to demonstrate the multilineage nature of the hCD45+, engrafted cells. Results for two representative animals; one that received untreated cells (top panel) and one that received the epinephrine treated cells (bottom panel).
of these neurotransmitters can be predicted to reduce their retention in haemopoietic niches.

Fresh and cultured CD34$^+$ cells were co-stained for CXCR4 and CD26 (receptors with important roles in migration, homing and engraftment) as well as $D_3$, $D_5$ and $\beta_2$. In fresh cells CXCR4 and CD26 expression was equally distributed between $\beta_2$ positive and $\beta_2$ negative cells. After cytokine stimulation, however, over 80% of the cells expressing these two receptors were also positive for $\beta_2$. The opposite effect was observed for dopamine receptors $D_3$ and $D_5$; cytokine culture increased expression of CXCR4 and CD26 but co-expression of the dopamine receptors within these sub-populations did not change. This is an interesting observation which suggests that, after culture, the cells that respond to SDF-1 gradient would have increased response to epinephrine (as was observed in the migration experiments) but not to dopamine. Thus they may, for example, respond to SDF-1 gradients but not to dopamine signals from the SNS. Moreover, if dopamine has a significant role in HSPC homing, as suggested by Spiegel and co-workers, these data could explain why homing is not increased in cytokine-cultured HSPC in vivo, despite the upregulation of CXCR4 and the increased SDF-1 directed migration, in vitro. Specific blockade of these receptors in in vivo experiments with fresh cells should be used to further investigate the importance of this observation.

The relationship between neurotransmitter expression and clonogenicity was examined before and after cytokine culture. Small subpopulations were selected according to their level of expression of each of the receptors and sorted into methylcellulose. For all three neurotransmitter receptors clonogenicity was not associated with the levels of expression on fresh cells.
After culture in cytokines, however, clonogenicity was significantly reduced in the cells expressing higher levels of the receptors. This effect was evident for all types of colonies produced and was statistically significant for all three receptors. This observation is particularly interesting, as it demonstrates that cells that are most responsive to SNS signalling lose their ability to produce colonies after culture in cytokines, potentially due to differentiation. *In vivo* this could mean that even if these cells home and are retained in the appropriate sites through SNS signalling they would not be able to reconstitute haemopoiesis. To confirm this hypothesis, further experiments should repeat this sorting protocol using LTC-IC assays as readout. This would exclude the possibility that the non-clonogenic cells are more primitive HSPC.

Cytokine cultured MPB CD34\(^+\) cells exhibit a significant reduction in short-term homing and long-term engraftment, which is associated with increased random migration and loss of adhesion to niche ligands. Here, an epinephrine pre-treatment protocol was utilised to modify the behaviour of cultured progenitors with the aim of improving their long-term engraftment. MPB CD34\(^+\) cells were cultured for three days in the standard cytokine cocktail with or without epinephrine (5nM). The effects of this treatment were tested in a variety of *in vitro* and *in vivo* assays.

Culture in epinephrine had no effect on the immunophenotype of the cells as expression of CD34, CD45 as well as \(\beta_2\), D\(_3\) and D\(_5\) did not change between treated and untreated cells. Moreover, the total and relevant co-expression of CXCR4 and CD26 with the neurotransmitter receptors is not altered by culture in epinephrine. Expression of several adhesion and homing receptors was similarly unaffected by epinephrine stimulation. Spiegel and colleagues demonstrated an increase of HSPC clonogenicity *in vitro* when the cells were stimulated with norepinephrine or dopamine agonists. Similar results have been previously reported for epinephrine by another group (Fonseca *et al.*, 2005). In support of these reports, *ex vivo* treatment with epinephrine was
found to increase clonogenicity of cytokine cultured HSPC, albeit moderately. This was significant for both myeloid and erythroid progenitors.

Haemopoietic progenitors have been shown to be selective in terms of their chemotaxis, mainly migrating towards SDF-1 (Peled et al, 2000; Wright et al, 2002). Spiegel et al demonstrated migration of HSPC towards norepinephrine and dopamine agonists. No equivalent data have been published for epinephrine. Freshly isolated MPB CD34+ cells do not appear to migrate towards epinephrine. After culture in cytokines, however, migration to epinephrine quadruples to reach levels significantly higher than baseline migration. This is consistent with the cytokine-induced upregulation of β2. In cultured cells, addition of epinephrine to SDF-1 does not further increase migration levels, which means that not all of the β2 expressing cells are able to induce chemotaxis. HSPC that were cultured in cytokines with the addition of epinephrine had similar responses to SDF-1 and the SDF-1/epinephrine combination but did not migrate towards epinephrine alone. Culture with epinephrine does not alter the expression levels of β2 but it may desensitise the receptor rendering it non-responsive to subsequent exposure in the migration assay.

Culture of CD34+ cells in cytokines in the presence of epinephrine (5nM) for 72 hours had a significant impact on the levels of HSPC homing, compared with cytokine culture alone. This was an interesting observation with potential clinical applications. The epinephrine-induced increase in homing levels is not associated with an upregulation of several associated receptors (namely CD44, CD49D, CD49E and L-selectin), although upregulation of other receptors cannot be excluded from these experiments.

An alternative explanation for this increase in homing could be related to the β-catenin pathway. Spiegel and colleagues have described that culture in epinephrine induces β-catenin accumulation (Spiegel et al, 2007) which in
turn increases proliferation and self-renewal (Reya et al, 2003). In addition to its role in HSPC proliferation and clonogenicity, this pathway has also been shown to affect homing to the BM. Blockade of the Wnt pathway (with Dickkopf-1) has been shown to induce HSPC mobilisation (Spiegel et al, 2007; Aicher et al, 2008) and to cancel the positive effect of neurotransmitter treatment on HSC engraftment (Spiegel et al, 2007). At the same time, other treatments that cause β-catenin accumulation, such as prostaglandin E2 (Wang et al, 2004), also significantly increase homing of HSPC to the BM (Hoggatt et al, 2009). It is, therefore, possible that epinephrine stimulation of cultured HSPC induces β-catenin accumulation on these cells which, in turn, leads to increased BM homing.

Additionally, cultured cells demonstrated increased β2 receptor expression and (subsequently) a chemotactic response towards epinephrine that was not seen by fresh cells. It is, therefore, possible that the increased migration towards epinephrine, exhibited by cultured cells, might be associated with non-specific homing to epinephrine gradients in non-haemopoietic tissue. Desensitisation of the receptor (with epinephrine treatment) potentially corrects this effect thus improving specific homing.

The loss of adhesion to specific BM ligands, leading to loss of attachment within the cavities, has been previously discussed as a potential contributing factor in cytokine-induced loss of engraftment (Chapter 6). The effect of epinephrine treatment on the adhesion of cultured HSPC was tested in adhesion assays to assess whether this protocol can modulate this phenomenon. The length of exposure of the cells to the neurotransmitter was optimised and 24 hours was found to be sufficient to induce an effect on N-cadherin adhesion.

Interestingly, using this protocol, adhesion of cultured cells to N-cadherin and osteopontin was significantly increased, reaching the levels achieved by fresh
progenitors. No effect was observed for adhesion to VCAM-1. The receptors that mediate adhesion to these ligands (N-cadherin, CD44 and VLA-4), however, were not upregulated with the epinephrine pre-treatment. Epinephrine excreting nerve endings are found in close proximity to osteoblasts and osteoclasts in vivo where HSC reside and sympathetic signalling has been shown to control the bone-remodelling process which controls the retention/release of HSC from the BM (reviewed in Lapidot & Kollet, 2010). It can be assumed, therefore, that SNS signals preferentially act on HSC located in the osteoblastic niches. The fact that epinephrine treatment affects adhesion to the endosteal niche ligands (N-cadherin and osteopontin) but not the vascular one (VCAM-1) is consistent with these observations. B-catenin is a structural adaptor protein that forms complexes with cadherins by binding to their cytoplasmic domain and linking them to the actin cytoskeleton (Gumbiner, 2000; Jamora & Fuchs, 2002). This process directly affects cell-cell adhesion. If epinephrine induces β-catenin accumulation that could, in turn, alter the N-cadherin function which results in increased homotypic adhesion.

To further validate this data, the same treatment was used for cultured HSPC infused in the ex vivo attachment model. In this context, epinephrine pre-treatment increased HSPC attachment but the difference was not statistically significant. This moderate increase in cultured HSPC attachment in the rat model could be related to the fact that this treatment causes a specific, but not global increase in adhesion, as shown in the in vitro adhesion assays.

Stimulation of cytokine cultured cells with epinephrine significantly increased the resulting engraftment. This finding is potentially clinically interesting and thus further work should aim to dissect the pathways activated after epinephrine treatment of cytokine cultured cells and to test its safety as an ex vivo treatment of HSPC pre-transplantation.
9 Conclusions

A: Effects of cytokine culture on MPB CD34+ cells

This work aimed to describe the effects of cytokine culture on MPB HSPC and to explore strategies to modulate these cells in order to improve their behaviour after culture.

Culture of MPB CD34+ cells in cytokines for 72 hours was shown to induce cycling, leading to expansion of the population. In addition to this, clonogenicity was increased while LTC-IC content was retained. Viability and CD34+ expression did not significantly change whereas CXCR4 expression increased. The ability of the cytokine cultured cells to migrate, either randomly or towards an SDF-1 gradient, was significantly increased, in comparison to uncultured cells. Despite this, the short-term homing of cultured cells in immunodeficient mice is decreased, which is at least partly responsible for the subsequent loss of long-term engraftment in these animals.

Following IV infusion of HSPC in recipient animals significant numbers of cells are lost in non-haemopoietic tissue and fail to reach the BM cavities. Bypassing this inefficient homing process, by direct injection of HSPC into the BM cavities of immunodeficient mice (IB transplantation), significantly increased the resulting engraftment, compared to IV infusion. When IB injection was used to deliver the cultured HSPC to the conditioned animals, however, the levels of engraftment were substantially lower than those achieved by their fresh counterparts. Cytokine culture appeared to decrease the long-term retention of these cells at the site of injection, while their ability to transmigrate and colonise other haemopoietic tissue was also reduced.

To further investigate the attachment defect exhibited by HSPC after culture in cytokines, a novel ex vivo BM model was developed, using the long bones
from young rats. In this model, cytokine activation significantly reduced the levels of HSPC attachment, confirming the observations from the IB transplantation experiments.

*In vitro* adhesion assays demonstrated that the specific adhesion of freshly isolated MPB progenitors to N-cadherin, osteopontin and VCAM-1, putative ligands of the osteoblastic and vascular niches, was significantly decreased following cytokine exposure. This was not, however, associated with loss of receptors or lower mRNA levels. More likely, exposure to cytokines is likely to induce post-translational, conformational modifications to these receptors which render them less responsive to the ligands in the niches.

Taken together, these findings indicate that cytokine culture of MPB HSPC induces an attachment defect which reduces their retention in the BM. This effect is not global (as it was not observed for adhesion to fibronectin) but is related to specific niche ligands and is not caused from downregulation of the relevant receptors on the HSPC after culture. The effect of cytokine exposure on HSPC adhesion ligands has been previously studied in different models and assays, but no specific trend was described that could explain the reduction in engraftment. This work, confirms some findings previously published and comprehensively describes the attachment defect of cytokine cultured cells but it does not offer a mechanistic explanation for this effect.

The rat model described here was developed to answer specific questions regarding the interaction of HSPC with the BM surface. It can be argued that rat models are not regularly used in transplantation models. They have, however, been used to study HSPC behaviour in other disease models. The rat adhesion ligands and receptors share great homology with their human counterparts and thus cross-reactivity can be assumed, at least to the levels seen in the well-studied mouse models. The flushing process (prior to human HSPC infusion) will certainly change the architecture of the BM cavity and will
also remove some vascular elements. This process is necessary in order to vacate the resident HSPC. The bone surface and extracellular matrix proteins are expected to remain after the flushing process. This was partly shown in the blockade experiments, where HSPC adhesion in the bones was reduced after inhibition of specific receptors which bind to ligands present in the osteoblastic niches and extracellular matrix. Thus, despite the disrupted vascular surface this model remains a useful platform on which HSPC attachment can be studied in several contexts. Finally, pre-irradiation of the animals is also known to disturb the architecture of the BM. However, this conditioning treatment is regularly used in clinic and in the established mouse transplantation models. It also increases SDF-1 production from osteoblasts which enhances HSPC adhesion. Overall, this novel model is a valuable and valid tool to describe HSPC behaviour in the BM niches.

**B: Modulations to improve the function of cultured HSPC**

CD26 is a peptidase which targets SDF-1 inhibiting its chemotactic effect on CXCR4. Blockade of CD26 has been used as a method to improve transplantation of fresh and *ex vivo* cultured murine and human CB HSPC. In this work no significant effects were seen on the migration and adhesion of MPB HSPC *in vitro*, after CD26 inhibition with diprotin A.

CD26 inhibition on cultured HSPC moderately increased homing but did not improve long-term engraftment of these cells in immunodeficient mice. *Ex vivo* treatment combined with *in vivo* infusion of diprotin A at the time of transplantation rescued the short-term homing of cultured HSPC while it increased long-term engraftment by 1.5-fold. This protocol inhibits CD26 expressed by endothelial cells in the recipient and can therefore, render the transplanted cells more sensitive to SDF-1 gradients. While the combined protocol provides a transplantation advantage and could potentially be
translated in clinic, the proteolytic activity of CD26 does not appear to be responsible for the cytokine-induced engraftment defect.

Mounting evidence suggests that neurotransmitter signaling regulates not only the release of HSPC from their niches but also their proliferation in these sites. MPB HSPC express the adrenergic receptor $\beta_2$ as well as the dopamine receptors $D_3$ and $D_5$. Cytokine exposure increases expression of $\beta_2$ whereas the levels of $D_3$ and $D_5$ are downregulated. Interestingly, the clonogenicity of the cells positive for all three receptors is significantly decreased after culture in cytokines in comparison to their negative counterparts. These observations demonstrate a previously unreported effect of cytokine culture on neurotransmitter receptors expressed on HSPC, with potentially significant consequences on their function and responsiveness to SNS signals, in vivo.

Epinephrine stimulation increased the adhesion of cultured progenitors to N-cadherin and osteopontin in vitro. In vivo, pre-treatment of cultured HSPC with epinephrine significantly increased the homing of these cells to the BM of $\beta_2^{m/NOD/SCID}$ mice. Additionally, a 2-fold increase in long-term engraftment of cultured HSPC was seen after epinephrine stimulation. This epinephrine-induced effect on cytokine cultured HSPC homing and engraftment has not been previously investigated and has interesting clinical possibilities. Epinephrine pre-stimulation could be used to increase the engraftment of cultured HSPC in situations where limited cell numbers are available for HSCT.

**Future directions**

Cytokine culture was shown to induce a specific adhesion defect which is not related to the expression levels of the respective receptors on the HSPC. Conformational and functional changes, such as lipid raft clustering, could be the cause of this effect. Further work in this aspect of the work should aim to identify and correct these conformational changes. To investigate the
involvement of lipid rafts in particular, the distribution of this membrane
component as well as the associated receptors could be investigated before
and after culture in cytokines. Provided there is a lipid raft re-arrangement
after exposure to cytokines, compounds such as methyl-β-cyclodextrin could
be used to maintain the lipid raft status of fresh cells and prevent the
clustering which may be inducing the functional changes we have observed
here.

Moreover, Rho GTPases and the Rac subfamily in particular, have been
associated with HSPC function. Rac1 and Rac2, specifically appear to directly
mediate the homing, migration and adhesion properties of HSPC through
“Inside-out” signaling. Retention of these cells in the BM is significantly
reduced in Rac2−/− mice (Yang et al, 2001). Future experiments should
investigate expression of Rac2 in cytokine cultured HSPC and attempt to
modulate this pathway, to enhance the resulting BM attachment. Induced
expression of Rac2, with lentiviral vectors and inhibition with RNA
interference or small molecule inhibitors, for example, are some of the
recently developed protocols for GTPase modulation that could be utilised in
this context.

CD26 inhibition with the combined diprotin A protocol was shown to improve
homing and increase long-term engraftment of cultured HSPC. Engraftment
levels of cultured cells in β2m/NOD/SCID mice, however, were comparatively
high, potentially masking the diprotin A effect. Further experiments should test
for the engraftment of limiting numbers of cultured cells with and without the
combined treatment. Additionally, as diprotin A could be targeting additional
receptors, a more specific approach, using RNA interference, could be used
to test the effects of CD26 inhibition in these models.

In Chapter 8, a previously unreported effect of epinephrine pre-stimulation on
the adhesion, homing and long-term engraftment of cultured MPB HSPC is
described. The molecular pathways affected by epinephrine stimulation on HSPC, however, were not investigated. Spiegel and co-workers suggest an involvement of the Wnt pathway as accumulation of β-catenin was observed in the neurotransmitter stimulated HSPC. Future work in this section should aim to elaborate these factors, starting with blockade of the Wnt pathway (with Dickkopf-1). Also, β-catenin has been shown to form complexes with cadherins and to mediate their function. Confirming this interaction in epinephrine stimulated, cytokine-cultured HSPC would provide a mechanistic explanation for the increased N-cadherin adhesion observed. Interestingly, Rac1 GTPase has been shown to form complexes with β-catenin, promoting phosphorylation and nuclear translocation of the latter that bypasses the Wnt-dependent activation pathway. It would, therefore, be interesting to study the role of this GTPases in combination with epinephrine stimulation, which induces β-catenin accumulation.

Additionally, engraftment experiments in the β2m/NOD/SCID model with epinephrine pre-treatment should be performed, with limiting numbers of CD34+ cells (for reasons discussed above) and using fresh epinephrine stimulated and non-stimulated cells for comparison.

As part of this project, a novel, ex vivo attachment model was developed, and used to test progenitor attachment in the BM after cytokine culture or to evaluate the effects of CD26 inhibition and epinephrine stimulation in this context. Further uses of this model could involve testing of other expansion strategies in terms of their effect on BM attachment. Similarly, cellular treatments of the expanded cells (prior to transplantation) could also be tested. Moreover, pre-treatments of the recipient (such as niche expansion protocols using parathyroid hormone or bone anabolic agents) can be tested with this model, to investigate their effect on HSPC retention. Additionally, imaging of the bones with the infused cells ex vivo, which would be less demanding than the equivalent process in vivo, could give more specific
information regarding the sites where HSPC attach. Overall, this model provides a platform on which specific questions can be answered regarding the interaction of HSPC with the BM surface, in an environment more representative than that of the \textit{in vitro} adhesion assays and more controlled than that of \textit{in vivo} engraftment studies.
### Appendix

#### 1. Plastics and tissue culture materials

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<td>Bone wax</td>
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Polycarbonate membranes (Transwells, migration) pore size 3.0µm, diam. 6.5mm Corning (Sigma) CLS3415-48EA

### I.2 CD34 cell selection reagents

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<td>hCD34 Microbeads</td>
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### I.3 Cell culture reagents

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### I.4 Cytokines, adhesion ligands and other proteins

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### I.5 Inhibitors, peptides and primers

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<td>555751</td>
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<td>anti-rabbit APC</td>
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<tr>
<td>Monoclonal anti N-cadherin antibody</td>
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<td>Sigma</td>
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<td>FITC anti hCD62L (L-Selectin)</td>
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<td>PE anti hCD49e</td>
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### 1.7 Dyes kits and chemicals

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<tr>
<td>0.4% Trypan Blue</td>
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<td>Sigma</td>
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<td>Propidium Iodide</td>
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<td>Sigma</td>
<td>P4170</td>
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<td>Kit for RNA isolation</td>
<td>50 samples</td>
<td>Qiagen</td>
<td>79254</td>
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<tr>
<td>Reverse transcriptase</td>
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<td>Life technologies (Invitrogen)</td>
<td>18080044</td>
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<td>(Superscript III)</td>
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<td>SYBR Green</td>
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<td>Sigma</td>
<td>S4438-100RXN</td>
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<td>Glycerol</td>
<td>1lt</td>
<td>VWR</td>
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<td>Bromophenol blue</td>
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<td>Merck</td>
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<td>Ethidium bromide</td>
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<td>Sigma</td>
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<td>Quantity</td>
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<td>EDTA</td>
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<td>VWR</td>
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<td>Tris base</td>
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<td>Boric acid</td>
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<td>VWR</td>
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<td>Agarose</td>
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<td>Bioline</td>
<td>BIO-41025</td>
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### 1.8 Equipment and instruments

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<th>Model and Company</th>
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<tr>
<td>C0₂ Incubator</td>
<td>Galaxy R, Wolf Laboratories</td>
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<td>Centrifuges</td>
<td>Multifuge S-R, Heraeus</td>
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<td></td>
<td>Centrifuge 5804, Eppendorfs</td>
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<tr>
<td>Class II Microbiological safety cabinet</td>
<td>Walker safety cabinets Ltd, UK</td>
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<tr>
<td>Flow cytometers:</td>
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<tr>
<td>Cyan ADP</td>
<td>Beckman Coulter, UK</td>
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<td>MoFlow XPD</td>
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<tr>
<td>Mastercycler ep Realplex</td>
<td>Eppendorf</td>
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<tr>
<td>Inverted microscope</td>
<td>DX61, Vision Biomed</td>
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<td>Labophot 2, Nikon</td>
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<td>Irradiator</td>
<td>Gammacell CIS IBL 437</td>
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<td>Dissection microscope</td>
<td>223327, Olympus</td>
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<tr>
<td>Animal restrainer</td>
<td>Harvard Apparatus</td>
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<tr>
<td>Haemocytometer</td>
<td>VWR</td>
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<tr>
<td>Bone-cutters</td>
<td>S Murray &amp; Co</td>
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<td>Dissecting Forceps</td>
<td>SLS LTD</td>
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<td>Dissecting scissors</td>
<td>Sigma</td>
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<tr>
<td>Waterbath</td>
<td>JB Aqua 12 Plus, Grant</td>
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**I.9 Software**

<table>
<thead>
<tr>
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<tr>
<td>GraphPad Prism</td>
<td>GraphPad Software, Inc</td>
</tr>
<tr>
<td>Summit v4.3 &amp; v5.1</td>
<td>Dako Colorado, Inc</td>
</tr>
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</table>

**I.10 Buffers and solutions**

**Freeze mix**
- DMSO 1ml
- FCS 4ml
- Prepared fresh and filtered before use. Added to cells resuspended in FCS at 1:1 proportion

**PI cell cycle staining solution**
- Propidium iodide 2mg/ml in ddH20 200µl
- RNase 10mg/ml in 10mM Tris (pH 7.5) 500µl
- 15mM NaCl
- PBS w/o Ca$^{2+}$ Mg$^{2+}$ 10ml
- Filtered before use and stored at 4°C.

**RBC lysis buffer**
- NH$_4$Cl 8.3g
- NaHCO$_3$ 1.68g
- EDTA 0.4g
Made up to 1000ml with ddH₂O, filtered solution through a 0.2µM 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

Prepared fresh before use and filtered through a 0.2µM filter.

**Staining buffer**

- PBS w/o Ca²⁺ Mg²⁺ 49ml
- BSA 5% (w/v) 1ml

**Blocking buffer** (adhesion assays)

- PBS with Ca²⁺ Mg²⁺ 40ml
- BSA 5% (w/v) 10ml

**Wash buffer**

- PBS w/o Ca²⁺ Mg²⁺ 40ml
- FCS 10ml

**Transfer medium**

- RPMI 44ml
- FCS 5ml
- Heparin 1000U/ml 1ml

**5% BSA solution**

- BSA 2.5g
- PBS w/o Ca²⁺ Mg²⁺ (make up to) 50ml
10% Glucose solution
Glucose 5g
PBS w/o Ca<sub>2+</sub> Mg<sub>2+</sub> (make up to) 50ml

Overnight medium for thawed hCD34<sup>+</sup> cells
HSA solution (20%) 1ml
X-VIVO 10 4ml

10x TBE Buffer
Tris base 108.9g
Boric acid 55.7g
EDTA 7.4g
Made up into 1 litre of ddH<sub>2</sub>O

5x Loading buffer
Glycerol 30%
Bromophenol blue 0.025%
Made up in 1x TBE buffer

Running buffer
1x TBE
Ehtidium bromide 0.1µg/ml

2% Agarose gel
Agarose 2% (weight/volume)
TBE 35ml
Ethidium bromide (1mg/ml) 3.5µl
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