A COMPARATIVE QUANTITATIVE STUDY OF HUMAN MYELOID DENDRITIC CELL PROGENITORS IN CORD BLOOD, BONE MARROW, PERIPHERAL BLOOD AND THEIR MOBILIZATION KINETICS IN THE PERIPHERAL BLOOD OF CANCER PATIENTS UNDERGOING LEUCAPHAERESIS

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Contents

Chapter 4: A Comparative Study of CFC-DC in BM, CB, Mobilized PB and AC133 and CD33 Expression on $CD34^+$ Cells

Abstract

Dendritic cell (DC)-based immunotherapy has potential for use in the treatment of cancer, infections and transplantation. Generating large numbers of DC from haemopoietic progenitor cells (HPC) is a key step in this process, often achieved by the aphaeresis of HPC, following their mobilization from the bone marrow into peripheral blood (PB) with chemotherapy and growth factor support (usually, granulocyte-colony stimulating factor, G-CSF). The objective of this work was to identify the optimum time for leucaphaeresis of DC progenitors.

An established clonogenic assay specific for colony-forming cell (CFC) DC was used and validated with linearity, dose-response experiments and morphological confirmation. The optimal numbers of mononuclear and $CD34^+$ (or $AC133^+$) cells for plating were $5x10^4$ and $1\text{-}2x10^3$ respectively. The optimal concentrations of recombinant cytokines were also determined. Kinetic studies were done in patients with solid tumours, and HPC mobilization was achieved with conventional chemotherapy and G-CSF. The best time for harvesting large numbers of DC progenitors was when the leucocyte count rose rapidly from its nadir at a median 10 days (range 7-13) post chemotherapy.

Comparative studies identified mobilized PB as the richest source of CFC-DC (mean, 1,481/ ml PB), with at least, 1.5-fold more progenitors per unit volume than cord blood (CB). These data suggest that venesection alone could provide sufficient CFC-DC to generate mature DC, after *ex vivo* culture and expansion. This might obviate the need for leucaphaeresis thus making DC-based immunotherapy potentially more widely available. In all the haemopoietic tissues examined the majority of DC and granulo (G)-monocytic (M) progenitors, was found within the $CD34^+AC133^+$ cell population. It is concluded that the kinetics of mobilization of CFC-DC are very similar to those of other HPC like CFC-GM and erythroid progenitors. This has important implications for designing immunotherapy protocols to isolate DC precursors from $CD34^+$ HPC for ultimate use in DC-based immunotherapy.

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Leicester or any other University or Institute of learning

Dedication

This Thesis is Dedicated to

All The Cancer Patients that have Made it Possible

To Complete this Work

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Abbreviations (alphabetical)

ACDU	Automated cell deposition unit
AICD	Activation-induced cell death
Ag	Antigen
APC	Antigen presenting cell/s
AT	Adriamycin/ taxotere
BCG	Bacille of Calmette and Guerin
BEP	Bleomycin/ etoposide/ cisplatin
BFU-E	Burst forming unit-erythroid
BG	Birbeck granules
Bl-CFC	Blast colony forming cell
BM	Bone marrow
BMT	Bone marrow transplant
CB	Cord blood
CBT	Cord blood transplant
CD	Cluster of differentiation
CD40-L	CD40 ligand
CEPs	Circulating endothelial precursors
CFC-Bas	Colony forming cell-basophil
CFC-DC	Colony forming cell-dendritic cell
CFC-Eos	Colony forming cell-eosinophil
CFC-G	Colony forming cell-granulocyte

CFC-GEMM (Mix)	Colony forming cell-granulocyte erythroid macrophage megakaryocyte
CFC-GM	Colony forming cell-granulocyte macrophage
CFC-GMDC	Colony forming cell-granulocyte macrophage dendritic cell
CFC-M	Colony forming cell-macrophage
CFC-Meg	Colony forming cell-megakaryocyte
CFU-E	Colony forming unit-erythroid
CFU-S	Colony forming unit-spleen
CHOP	Cyclophosphamide/ adriamycin/ vincristine/ prednisolone
CLA	Cutaneous leucocyte homing antigen
CM	Conditioned medium
CSF/CSFs	Colony-stimulating factor/s
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBDIS-1	European breast cancer dose intensity study-1
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
Epo	Erythropoietin
FACS	Fluorescence-activated cell sorting
FEC	5-Fluorouracil/ epirubicin/ cyclophosphamide

FITC	Fluorescein isothiocyanate
FLT-3	Fetal liver tyrosine kinase receptor-3
FLT-3L	FLT-3 ligand
FXIIIa	Factor XIIIa (activated)
G	Granulocyte
GCDC (FDC)	Germinal centre (Follicular) dendritic cells
G-CSF	Granulocyte colony stimulating factor
GCT	Germ cell tumours
GM-CSF	Granulocyte macrophage colony stimulating factor
GM-CSFRα	GM-CSF receptor alpha
gp	Glycoprotein
GVHD	Graft versus host disease
HDCT	High-dose chemotherapy
HGF/HGFs	Haemopoietic growth factor/s
HME	Haemopoietic microenvironment
HPC	Haemopoietic progenitor cells
HPP-CFC	High proliferative potential colony forming cell
HSC	Haemopoietic stem cells
ICAM	Intercellular adhesion molecule
i/d	Intradermal
IDGDC	Interdigitating dendritic cells
IFN-α/β/γ	Interferon-alpha/beta/gamma

Ig/s	Immunoglobulin/s
IL	Interleukin
IL-3Rα	Interleukin-3 receptor alpha
IMDM	Iscove's modified Dulbecco's medium
INTDC	Interstitial dendritic cells
IPC/s	Interferon producing cell/s
ISHAGE	International Society for Graft Engineering and Haematotherapy
iv	Intravenous
Lag	Langerin
LC/LCs	Langerhans cell/s
LFA	Leucocyte functional antigen
LP	Leucaphaeresis (aphaeresis) products
LPS	Lipopolysaccharide
LTBMC	Long-term bone marrow culture
LTCIC	Long-term culture-initiating cell
M-CSF	Macrophage colony stimulating factor
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MIP-1α/ 3β etc.	Macrophage inflammatory protein-1 alpha/ 3 beta
MIICs	MHC class II compartments
MNC	Mononuclear cells
Mo	Monocyte

MoAb	Monoclonal antibody/ies
MoCM	Monocyte-conditioned medium
Mo-DC	Monocyte-derived dendritic cells
MRA	Marrow repopulating activity
mRNA	Messenger RNA (ribonucleic acid)
N/A	Not available
N/E	Not evaluable
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cell
NOD	Non-obese diabetic
PAP	Prostatic alkaline phosphatase
PB	Peripheral blood
PBPC	Peripheral blood progenitor cells
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cells
PBPCT	Peripheral blood progenitor cell transplant
PBSCT	Peripheral blood stem cell transplant
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PSMA	Prostate specific membrane antigen
R	Receptor
r-	Recombinant

rh-	Recombinant human (for cytokines)
s/c	Subcutaneous
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDF-1	Stroma-derived factor 1
SEM	Standard error of the mean
SRCs	SCID repopulating cell/s
TCR	T-cell receptor (CD3)
TGF-β	Transforming growth factor-beta
THYMDC	Thymic dendritic cells
TNF-α	Tumour necrosis factor alpha
Tpo	Thrombopoietin
tyr	Tyrosinase
UPN	Unique patient number
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VIP	(VP-16) Etoposide/ Ifosfamide/ Cisplatin
VLA	Very late antigen

Chapter 1

Introduction

1.1 Historical background

In 1990 a paper in the journal "Blood" by Reid and colleagues demonstrated, using a semisolid culture system, that dendritic cells (DC) shared a common progenitor with granulocytes and macrophages (Reid et al. 1990). Two years later in an elegant paper in "Nature" Caux and colleagues (Caux et al. 1992) showed that Langerhans type cells could be generated from umbilical cord blood (CB) $CD34^+$ haematopoietic progenitor cells (HPC), following culture with granulocyte macrophage-colony stimulating factor (GM-CSF) and tumour necrosis factor alpha (TNF-α). That study was a major breakthrough in this field. By 1994 James Young and co-workers from the Memorial Sloan-Kettering Cancer Centre (MSKCC) established a clonogenic assay making it possible to enumerate DC progenitors (Young et al. 1995).

The work in this thesis is based on the use of these semi-solid culture methods to enumerate DC progenitors, using a standardised assay, in all the clinically important sources of HPC i.e. Bone Marrow (BM), CB and mobilized PB. The last part of the thesis involves a study of the kinetics of mobilization, from BM into PB, of the progenitors that give rise to one of the most important cell type in the human body; the Dendritic cell. In this Introduction I will discuss some of the important aspects of haemopoiesis (section 1.2). A comprehensive account on DC biology will follow (section 1.3) and then, a discussion on the characterization of Blood Stem Cells (section 1.4), their mobilization from BM into PB and peripheral blood progenitor cell (PBPC) transplantation (section 1.5). Finally I will outline the objectives of this project (section 1.6).

1.2 The haemopoietic system

1.2.1 Introduction - The haemopoietic hierarchy

Haemopoiesis is the production of mature functional blood cells from committed progenitor cells, which are in turn thought to derive from pluripotent stem cells (Abramson et al. 1977; Ford et al. 1956; Wu et al. 1967). These cells give rise to progeny, which increase in numbers in succeeding compartments (Fig 1.2a). In the human as many as 10^{13} cells are produced in one day to replace cells of the myeloid, erythroid and lymphoid lineages (Allen et al. 1988). The production of such a large number of cells requires the presence of a haemopoietic hierarchy where pluripotent cells give rise to progeny to maintain homeostasis. So there is a continuum of cell types, which mature progressively while at the same time they lose their multipotentiality, and ability to repopulate the haemopoietic system of serially transplanted animals (Watt and Visser 1992).

Haemopoiesis is thought to start in the yolk sac (YS) (Moore and Metcalf 1970; Moore and Owen 1967) where non-haemopoietic precursors form blood islands (Figs 1.2b). Cells from these blood islands migrate to the fetal liver (Moore and Metcalf 1970) and are subsequently replaced by a second population of haemopoietic cells that arise *de novo* from non-haemopoietic progenitors in the para-aortic region of the fetus (Metcalf 1988). It is these cells that replace the YS-derived blood cells in the liver and then populate the fetal spleen and bone marrow. The change of the fetal liver as the site of haemopoiesis instead of the YS marks the onset of definitive multilineage haemopoiesis (Metcalf and Moore 1971).

Fig 1.2a The Basic Compartments of the Haemopoietic System. Modified from (Metcalf 1999).

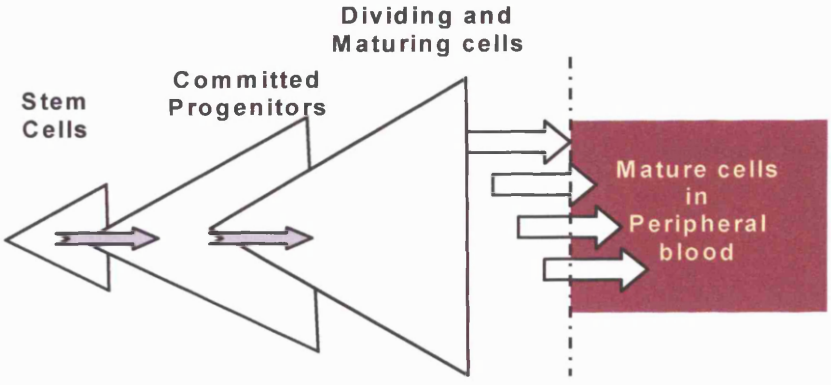
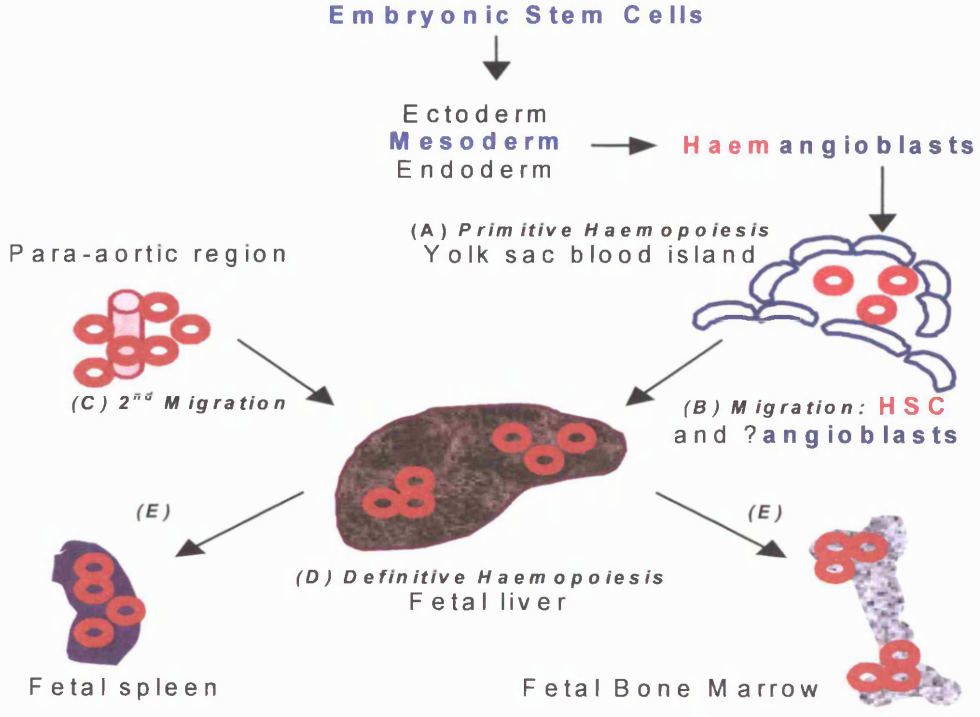


Fig 1.2b Early Development of Haemopoietic Organs in the Fetus.



(A) Haemopoiesis starts in the YS blood islands; (B) Haemopoietic cells migrate from the YS to the fetal liver; (C) Second wave of migration of Haemopoietic cells from the para-aortic region to the fetal liver; (D) Definitive haemopoiesis takes place in the fetal liver; (E) Haemopoietic cells in the liver migrate to the fetal spleen or BM. Adapted from (Metcalf 1988; Moore 1999; Robertson et al. 1999).

After birth the number of haemopoietic cells in the liver and spleen declines quickly and for the rest of the lifespan in humans, haemopoietic capacity is found in the BM scattered throughout the body (Metcalf 1988). The capacity of the liver/spleen to function as a haemopoietic organ is however maintained and if the BM becomes diseased extramedullary haemopoiesis in the liver and or spleen can take place (Metcalf and Moore 1971).

1.2.2 Stem cells

Stem cells are the most important cells of the haemopoietic system (Metcalf 1988). They are ultimately responsible for generating blood cells under steady state conditions for the normal life span of the individual, and also following myelosuppressive insult (e.g. cytotoxic or non-cytotoxic drugs, radiation, severe systemic illness) and after blood stem cell transplantation (Ford et al. 1956; Keller and Snodgrass 1990; Lemischka et al. 1986; Merwin 1959; Reiffers et al. 1986; Szilvassy et al. 1989). The study of stem cell biology is important because in addition to the above the development of defects in the stem cell DNA may result in abnormal progeny leading ultimately to lympho-myelodysplastic syndromes or haematological malignancies (Clark et al. 1986; Eaves and Eaves 1979; Lepine and Messner 1983; Papayannopoulou et al. 1978).

Pluripotent stem cells are a small sub-population in the peak of the haemopoietic hierarchy. They number ~ 1 per 100,000 bone marrow mononuclear cells (MNC) (Kvalheim and Smeland 1998; Metcalf and Nicola 1995). Phenotypically they are lymphocyte like cells i.e. small-medium mononuclear cells with large nuclear/cytoplasmic ratio. Stem cells with marrow repopulating ability are capable of extensive self-renewal; capacity for long-term lymphohaemopoietic organ

repopulation and can sustain long-term haemopoiesis (Dexter 1996; Metcalf and Nicola 1995). The original definition of stem cells was based on two functional criteria (Dexter et al. 1977; Till and McCulloch 1961):

1. The ability to self-renew.
2. The potential to differentiate into all lymphohaematopoietic lineages.

Stem cells are a heterogeneous group of cells because of the hierarchical relationship between the parental/ daughter cells within the stem cell population (Metcalf and Nicola 1995). Over the last 40 years functional assays have been developed to enumerate and study the different cell populations within the stem cell and progenitor compartments. A detailed account of some of the most important functional assays for haemopoietic stem cells (HSC) is given in section 1.4.

1.2.3 Committed progenitor cells

These are cells, which are committed usually to a single lineage of differentiation and less frequently to 2-3 lineages. They are characterized by the capacity to form colonies of maturing progeny in semi-solid culture assays (clonogenic assays) following stimulation with haemopoietic growth factors (please see section 1.4). The number of cells in these colonies can vary from 50 (usually the minimum cell number to define a colony) to several thousand (Metcalf and Nicola 1995). Committed progenitor cells are medium to large MNC, are usually in cell cycle and make up ~1% of haemopoietic cells. Most evidence suggests their differentiation commitment is irreversible (McArthur et al. 1995; McArthur et al. 1994). Such cells cannot revert back to multipotent stem cells (Metcalf 1999).

1.2.4 Dividing, differentiating and mature cells

These cells make-up the majority of blood cells. They are the progeny of more primitive cells and differentiate to mature blood cells. The process of maturation is characterized by changes in the size, shape of the cell, the nuclear cytoplasmic ratio, presence of cytoplasmic granules and other well-defined criteria (Metcalf and Nicola 1995). These features allow the cells to be distinguished from one another morphologically. As the cells mature their proliferative capacity becomes progressively restricted and ultimately lost. Fig 1.2c illustrates the terminal differentiation stages of granulocyte (G) and monocyte (Mo)/macrophage (M) committed progenitors. Following release from the BM into PB the mature cells enter the tissues. Monocytes undergo phenotypic changes and in the tissues are known as macrophages. Neutrophils extravasate to sites of inflammation where they play a major role against invading microrganisms. Similar stages of differentiation apply to all the other lineages.

1.2.5 The bone marrow stroma

The BM stroma is a network of cells and connective tissue, which make up the haemopoietic microenvironment (HME) (Charbord 1993). Dexter and colleagues demonstrated the importance of the BM stroma in the control of haemopoiesis very elegantly in 1977 with the development of the long-term BM culture (LTBMC) system (Dexter et al. 1977). The distinguishing features of this culture method from other haemopoietic assays are that, sustained haemopoiesis occurs in the absence of exogenous growth factors and the former depends entirely on the establishment of an adherent layer of BM-derived stromal cells. These stromal cells support haemopoiesis by providing an environment that allows for survival, self-renewal, proliferation and

differentiation of HSC (Allen et al. 1988). One of the key mechanisms involved is the intimate contact between HSC and stromal cells. The latter produce many cytokines (Fibbe et al. 1988; Gualtieri et al. 1984; Henney 1989; Yang et al. 1988), some of them, like stem cell factor (SCF) for example in soluble form. SCF can exist as membrane-bound (Anderson et al. 1990) and thus decreases the amount of colony-stimulating factor (CSF) needed for maximal cell proliferation by several hundred fold (Heyworth et al. 1992). Consequently very small concentrations of other cytokines can exert maximal biological effect (Heyworth et al. 1997).

The stromal cells have been described in the mouse and the human. The types of cells regularly found in normal BM are endothelial cells, adventitial reticular cells adipocytes, macrophages, endosteal cells and fibroblasts (Charbord 1993; Deryugina and Muller-Sieburg 1993). The stromal network is made up of:

1. A vascular network of sinuses, where mature cells egress into PB and particular material is removed from the circulation (Charbord 1993).
2. A connective tissue network made up of collagens (types I-VI), laminin, fibronectin and tenascin.

The stromal cells produce cell adhesion molecules (CAMs) (Whetton and Spooncer 1998) and glycosaminoglycans (Pettengell 1997), which together with collagens and other connective tissue proteins make-up the extracellular matrix (ECM). The ECM is vital in the maintenance of an adhesive HME. The interactions between the HSC and the BM stroma are complex as represented by Fig 1.2d.

Fig 1.2c Granulocyte and Monocyte Maturation (Metcalf and Nicola 1995).

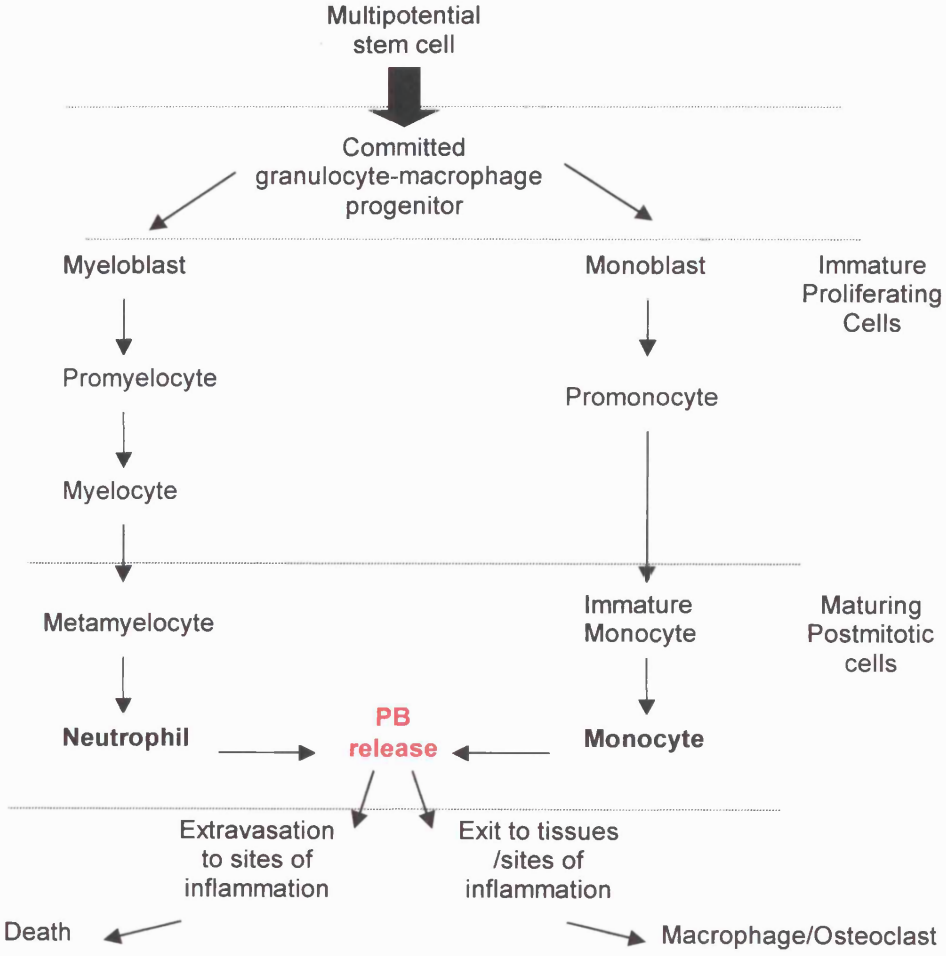
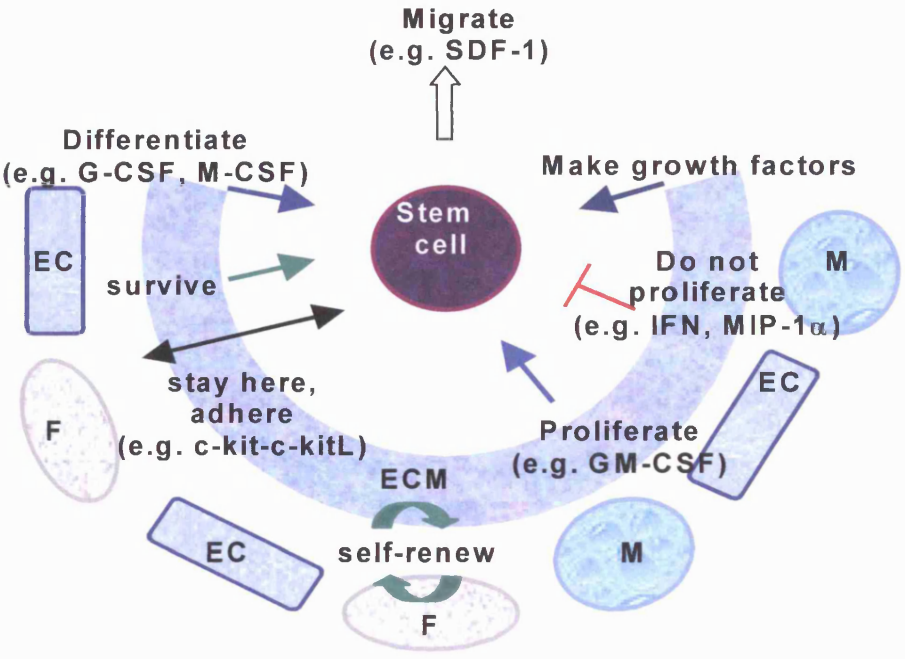


Fig 1.2d The Haemopoietic Microenvironment (HME) - Stem Cell Interactions.

Adapted and modified from (Moore 1991; Whetton and Spooncer 1998).



Responses of primitive HSC; e.g. "produce" vascular endothelial growth factor (VEGF)(Kennedy et al. 1997), "survive" in response to SCF, "migrate" in response to stroma-derived factor 1 (SDF-1), "adhere" via interaction between c-kit and c-kit ligand and integrins/ ECM molecules and "divide" or "do not divide" in response to cytokines (positive and negative regulators of haemopoiesis respectively). EC, endothelial cell; F, fibroblast; ECM, extracellular matrix; M, macrophage; c-kitL, c-kit ligand (also known as SCF).

1.2.6 Haemopoietic growth factors

Cohen introduced the term cytokine in 1974 for any soluble entity of lymphoid or non-lymphoid origin that exerted specialized effects on target cells (Cohen and Cohen 1996; Cohen et al. 1974). Because this was a broad term that would also include immunoglobulins (Igs), hormones, enzymes a more precise definition is that used by Meager as "a group of hormone-like polypeptide mediators that play a variety of regulatory roles in both host defense and normal and abnormal homeostatic mechanisms" (Meager 1988). Individual cytokines can have many different effects (i.e. pleiotropic) but also different cytokines can exhibit redundancy. The major groups of cytokines include colony-stimulating factors (CSFs), interferons (IFNs), inflammatory cytokines (e.g. TNF-α and -β, interleukin-1 -IL-1), lymphocyte-derived mediators (e.g. IL-2, IL-4), macrophage-derived cytokines (e.g. IL-12), chemokines and transforming growth factor-β (TGF-β) (reviewed in (Cohen and Cohen 1996)).

CSFs also known as haemopoietic growth factors (HGFs) are glycoproteins essential for the survival of HPC (Costa 1998; Nicola 1989). The main functional features of haemopoietic cytokines are, stimulation of proliferation in target cells, suppression of apoptosis and influencing HPC to undergo lineage commitment. In 1966 a group of researchers in Melbourne developed a semi-solid culture assay where murine HPC were immobilised in a gel matrix and their growth was supported with media conditioned by the growth of non-haemopoietic cells (Bradley and Metcalf 1966; Pluznik and Sachs 1966). Colonies of mature granulocytes were grown by this method and so the term colony-stimulating factor (CSF) was introduced, to encompass the factors that stimulated the growth proliferation and differentiation of the progenitor cells. The assay was used for human HPC (Chervenick and Boggs 1971; McCredie et al. 1971) and later applied to other haemopoietic lineages;

erythroid (Stephenson et al. 1971), megakaryocytic (Metcalf et al. 1975a), B lymphocytes (Metcalf et al. 1975b), and dendritic cells (Reid et al. 1990; Young et al. 1995).

DNA technology allowed the identification of genes coding for CSFs and subsequently their production in bacterial/ yeast/ mammalian cell expression systems (Nicola 1989). Among the first recombinant (r) CSFs produced were G-CSF, M-CSF, GM-CSF and IL-3 (reviewed by (Dexter 1996; Robinson and Quesenberry 1990a; Robinson and Quesenberry 1990b; Robinson and Quesenberry 1990c)). To date many more growth factors have been identified. Monoclonal antibodies (MoAb) and cytometric cell sorting have allowed the isolation of various progenitor populations thus making it possible to study the effect of HGFs on selected haemopoietic cell populations (Lord and Spooncer 1986; Spangrude et al. 1988; Visser et al. 1984). HGFs may be classified depending on the type of cell they exert their effects on. Hence there are early, intermediate and late-acting factors (Lowry 1995; Ogawa 1993). Their general characteristics and effects of their actions are shown in tables 1.2a-b. The cytokines used in this study were G-CSF, GM-CSF, Erythropoietin (Epo), SCF and TNF-α.

G-CSF: G-CSF was originally purified from mouse lung conditioned medium (Nicola et al. 1983). G-CSF (molecular weight 25-kDal, (Nagata et al. 1986; Nicola et al. 1985)) has effects on early progenitors, on differentiating cells of the GM pathway and on the functional capabilities of neutrophils (Nicola 1990). In clinical trials it has been shown to decrease the severity of chemotherapy-induced neutropenia (Bronchud et al. 1989; Crawford et al. 1991). It is commonly used to enhance mobilization of HPC from the BM into PB (reviewed by (To et al. 1997) and in section 1.5).

Table 1.2a. General Characteristics of HGFs (Robinson and Quesenberry 1990a).

Characteristics of Haemopoietic Growth factors (HGFs)
Glycoproteins, with molecular weights 18 to 90-kDal
Active at low concentrations *in vivo* and *in vitro*
Often produced by several cell types
Stimulate both proliferation and differentiation of target cells
Frequently synergistic with other growth factors
Produced constitutively or induced by various stimuli

Table 1.2b. Effect of CSFs on Haemopoietic Cells. Adapted and modified from (Lowry 1995; Ogawa 1993).

Factors	Activity
1. Early Factors: Stimulate cell division in dormant progenitors. Recruitment may need combined activation of at least 2 cytokine receptors	
Stem Cell Factors	SCF (c-kit ligand), Flt-3L (Flt-3 ligand)
CSFs	G-CSF, M-CSF, TPO (Thrombopoietin), IL-3
Synergistic factors	IL-1, IL-4, IL-6, IL-11, IL-12 and LIF (Leukaemia inhibitory factor)
2. Intermediate Factors: Support proliferation after multipotential progenitors exit G_0. Interact with late-acting factors to produce more mature cells, e.g. IL-3, IL-4, GM-CSF	
3. Late Factors: Lineage-specific action. Support proliferation and differentiation of committed progenitors	
EPO	Erythrocyte production
M-CSF	Macrophage differentiation and function
G-CSF	Granulocyte differentiation and function
IL-5	Eosinophil and B cell differentiation and Ig secretion
TPO	Platelet production

GM-CSF: GM-CSF was characterized from murine lung conditioned media in 1977 (Burgess et al. 1977) and cloned from a human leukaemic cell line in 1984 (Gough et al. 1984). It acts on early progenitor cells and cells committed to the GM (Lowry 1995) and DC pathway (Young 1999a). It has significant functional effects on neutrophils and monocytes/ macrophages such as phagocytosis, superoxide production, and antibody (Ab)-dependent cell-mediated cytotoxicity (Gasson et al. 1984; Sieff et al. 1985; Weisbart et al. 1985). It also enhances the function of antigen presenting cells (APC) like DC and macrophages (Armitage 1998). GM-CSF was the first r-HGF shown to increase the number of PBPC mobilized by chemotherapy (Gianni et al. 1989; Socinski et al. 1988) and the first one used to mobilize PBPC for auto transplantation (Gianni et al. 1989).

Erythropoietin: This was purified from human urine in 1977 (Miyake et al. 1977) and the gene was cloned in 1985 (Jacobs et al. 1985; Lin et al. 1985). The kidneys produce most Epo and production is increased in response to hypoxia although the precise mechanism is unknown (Robinson and Quesenberry 1990b). Epo is not just a red cell specific growth factor (Goldwasser et al. 1990); it exerts effects on granulocytes, macrophages and megakaryocytes *in vitro* and *in vivo* (Berridge et al. 1988; Dessypris et al. 1988).

SCF: SCF also known, as Steel factor or mast cell growth factor is a prototype of an early acting HGF. SCF, which was isolated, and its gene cloned in 1990, is the ligand for the proto-oncogene product c-kit, a transmembrane tyrosine kinase receptor, expressed predominantly on stem cells and progenitor cells (reviewed in (Lyman and Jacobsen 1998)). SCF and its cognate receptor are essential for haemopoiesis (Lyman and Jacobsen 1998). SCF works synergistically with intermediate and late acting factors and promotes marked proliferation of early HPC (Lowry 1995; Ogawa 1993)

including DC progenitors (Santiago-Schwarz et al. 1995; Young et al. 1995). The synergism of SCF with other HGFs has been exploited in the clinical setting where SCF and G-CSF enhance PBPC mobilization (please see section 1.5).

TNF-α: TNF-α was isolated in 1975 as a factor, present in the serum of BCG (Bacillus of Calmette and Guerin)-sensitized mice injected with endotoxin and which caused haemorrhagic necrosis of murine tumours *in vivo* (Carswell et al. 1975). TNF-α has a molecular weight of 17-kDal (Aggarwal 1992) and is a member of the TNF family of cytokines, which includes the apoptosis-inducing ligand Fas and CD40 ligand (Smith et al. 1994). TNF-α is a multifunctional cytokine with effects on several cell types. In the haemopoietic system TNF-α has bi-directional effects. TNF-α is an inhibitor of *in vitro* haemopoiesis while *in vivo* studies suggest it stimulates early HPC but inhibits late progenitors and in particular erythroid precursors (Trichieri 1992). The response of HPC to TNF-α depends on the stimulus used. For example BM-derived clonogenic progenitors stimulated *in vitro* with G-CSF are inhibited by TNF-α. However in combination with IL-3 and GM-CSF, TNF-α potentiated the proliferation induced by these two cytokines (Moore 1991).

Inhibitory cytokines: Interferons (IFNs), transforming growth factor-β (TGF-β) and macrophage inflammatory protein-1α (MIP-1α) are negative regulators of haemopoiesis (reviewed in (Graham and Wright 1997; Moore 1991). IFNs and TNF-α are lineage non-specific inhibitors, while TGF-β and MIP-1α affect more primitive cells (Ogawa 1993). Such inhibitory cytokines act as physiologic regulators of haemopoiesis (Ogawa 1993) but may also protect stem cells and more primitive progenitors from the effects of cytotoxic treatments (Veiby et al. 1997).

1.3 The Dendritic cell (DC)

1.3.1 Historical background

The Dendritic cell celebrated its 25th birthday in 1998. It is now 29 years since Ralph Steinman and Zanvil Cohn working in the Laboratory of Cellular Immunology and Physiology of the Rockfeller University in New York made this unique discovery. In the original paper in the Journal of Experimental Medicine, the DC was described as "a large stellate cell with distinct properties from those of mononuclear phagocytes, granulocytes and lymphocytes" (Steinman and Cohn 1973). In fact it was in 1868 when Paul Langerhans, an Austrian Medical Student, described in Virchows Archives the first population of DC in the epidermis (Langerhans 1868), which to date bear his name (Langerhans cells). Since Steinman's discovery a great deal has been achieved in this exciting field. It is beyond the scope of this section to describe all that is known about the DC. However I will discuss the most important aspects of DC biology particularly those relevant to haemopoiesis. Table 1.3a illustrates some of the most important milestones in this field.

Table 1.3a Important Advances in DC Biology. Landmark discoveries in bold

Discovery of Langerhans and Dendritic Cell (1868-1973)	
1868	**Paul Langerhans described DC in the skin** (Langerhans 1868)
1973	DC described in the spleen of mice by Steinman and Cohn (Steinman and Cohn 1973)
Origin of DC (1979-1990)	
1979	**BM origin of DC** (Katz et al. 1979)
1990	Common origin of DC with granulocytes and macrophages confirmed (Reid et al. 1990)
Identification of cytokine requirements and clinical use (1992- to date)	
1992	**GM-CSF and TNF-α promote the differentiation of $CD34^+$ cells into Dendritic-Langerhans cells *in vitro*** (Caux et al. 1992)
1995	Common lymphoid progenitor for DC identified (Galy et al. 1995)
1996-8	Clinical trials reporting responses in 3 out of 4 patients with low-grade lymphoma (Hsu et al. 1996) and 5 out of 16 patients with advanced melanoma (Nestle et al. 1998) following DC vaccination
2000	First clinical trial reporting remarkable regressions of metastatic renal cancer following vaccination with hybrid DC-tumour fusion vaccines (Kugler et al. 2000)

1.3.2 Definition and features of DC

Dendritic cells are "professional" antigen presenting cells (APCs), which are essential for the initiation of immune responses (Banchereau and Steinman 1998; Hart 1997; Steinman 1991). The word professional is used to denote the difference between DC and other types of non-professional APCs. Professional APCs express major histocompatibility complex (MHC) class II molecules and deliver co-stimulatory signals (see below). They differ in this respect from non-professional APCs, which can be induced to express class II molecules or deliver co-stimulatory signals. Examples of other professional APCs are macrophages and B cells. Non-professional APCs include fibroblasts, glial cells, pancreatic β cells, thymic and thyroid epithelial cells and vascular endothelial cells (Kuby 1997).

DC are BM-derived (i.e. from $CD34^+$ HPC) and make up a very small fraction of the whole leucocyte population. They account for 0.1-1% of MNC (Fearnley et al. 1999; Reid 1997). DC are a unique leucocyte population, characterized by typical dendritic morphology and high levels of MHC class II molecules (Caux and Banchereau 1996; Cella et al. 1997; Hart 1997; Inaba et al. 1997; Pierre et al. 1997; Winzler et al. 1997). DC have been defined using phenotypic and functional characteristics because of the absence of a unique marker such as CD3, CD14, CD16, CD19 or CD56 (i.e. lineage negative, Lin^-) and their heterogeneity; i.e. different types of DC found in different organs at different stages of development/ maturation. The following parameters are generally used to confirm the identity of a cell as dendritic (reviewed in (Hart 1997)):

1. Typical dendritic morphology; i.e. short or long ("veils") cytoplasmic projections (Fig 1.3a).
2. High levels of MHC class I and class II molecules.

3. High levels of co-stimulatory molecules (CD80 and CD86). These are essential for the stimulation of T lymphocytes by providing a second "activation" signal during the interaction between a T cell and a DC.

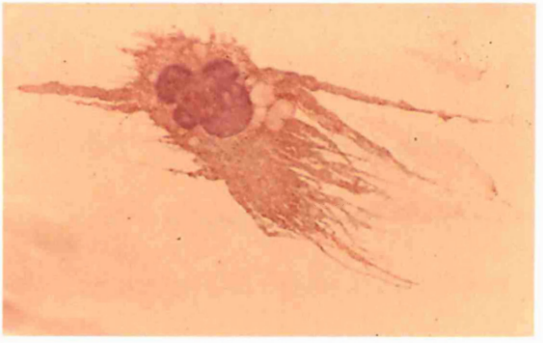
4. Potent primary T cell stimulatory activity.

5. Expression of high levels of cell adhesion molecules.

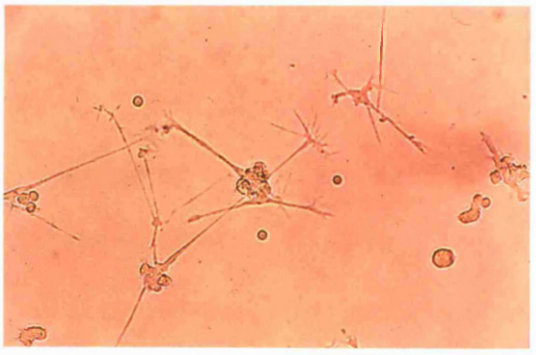
6. Other features include marked cell motility, phagocytic capability, expression of DC-associated antigens and spontaneous clustering with T cells *in vitro*.

The BM origin of DC was first demonstrated in transplant chimaeras in 1979 (Katz et al. 1979). Further indirect evidence supporting their BM origin in humans, comes from the expression of the common leucocyte antigen (CD45) in all DC and myeloid markers CD13, CD33 and CD11 on myeloid DC such as the epidermal Langerhans cells (Caux et al. 1999). $CD34^+$ DC progenitors migrate from the BM as circulating DC precursors and home into the tissues. There they reside as immature DC. After tissue damage they take up antigen and migrate to lymphoid organs where they initiate immune responses (Banchereau et al. 2000). DC are the only APCs which can induce primary immune responses and therefore allow establishment of immunological memory (Banchereau and Steinman 1998; Hart 1997; Steinman 1991). Follicular DC are found in the germinal centres (GC) of secondary lymphoid organs. Although they have dendritic morphology they are not bone marrow derived (CD45 neg). Unlike DC they present whole antigen (Ag) – antibody (Ab) complexes to B cells rather than processed Ag and are not included in the DC classification (Tew et al. 1999).

Fig 1.3a Morphological Characteristics of DC



A mature DC from mixed DC-GM colonies -derived after 14 days of culture- from CB $CD34^+$ cells plated in the CFC-DC assay. Immunohistochemistry confirms CD83 expression. Note the characteristic long veils and shorter dendrites as well as the multilobed nucleus. Original magnification x 1,000



Mature adherent DC within a pure DC colony that developed after 14 days of culture of $CD34^+$ cells derived from a leucaphaeresis product and plated in the CFC-DC assay. Original magnification x 250

1.3.3 The dendritic cell system- Pathways of DC development

DC make up an entire system. DC are heterogeneous with respect to anatomical distribution, precursor populations, function and outcome of the immune response (Banchereau et al. 2000). The precise relationship between these populations is still incompletely understood however it is thought they represent different stages of maturation (Steinman 1991). For example, clonogenic progenitors come from the BM, precursors circulate in PB and lymphatics, immature DC reside in peripheral tissues and mature DC are found in secondary lymphoid organs and the thymus (Young 1999a). The DC life cycle and the developmental pathways are illustrated in Figs 1.3b and 1.3c respectively. The phenotypic/ functional features of the different populations are summarised in table 1.3b. Animal and human studies (Caux et al. 1999) suggest DC subsets derive from myeloid (Reid et al. 1990; Young et al. 1995) and lymphoid clonogenic progenitors (Galy et al. 1995; Res et al. 1996) (Fig 1.3b).

(i) Myeloid DC: A $CD33^+$ myeloid progenitor (CFC-GMDC) identified in clonogenic assays (Reid et al. 1990; Young et al. 1995) differentiates into granulocytes, macrophages and DC (reviewed in (Peters et al. 1996; Young 1999a; Young and Steinman 1996)). Myeloid DC precursors in PB are of 2 types: $CD14^+CD11c^+CD1a^-$ monocytes (bipotential intermediate cells) which develop into either interstitial (tissue) macrophages in the presence of M-CSF, or Monocyte-derived DC (Mo-DC) in the presence of GM-CSF combined with IL-4 (Caux et al. 1999). This *in vitro* model of differentiation -without proliferation- of PB monocytes into Mo-DC was first described by Sallusto and colleagues (Sallusto et al. 1995; Sallusto and Lanzavecchia 1994) and subsequently by others (Chapuis et al. 1997; Romani et al. 1994; Romani et al. 1996; Zhou and Tedder 1996) and has been useful in studying the life cycle of human DC. Mo-DC can also develop along the same pathway from

$CD34^+$ HPC with GM-CSF and TNF-α (Caux et al. 1996a; Szabolcs et al. 1996). Mo-DC are phenotypically and functionally immature DC and can be induced to mature further by inflammatory stimuli (e.g. TNF-α, IL-1, CD40L) or Mo-conditioned medium (Mo-CM) (Reddy et al. 1997; Romani et al. 1996; van Kooten and Banchereau 1997; Zhou and Tedder 1996). $CD14^-CD11c^+CD1a^+$ precursors on the other hand differentiate into Langerhans cells (LC) under the influence of GM-CSF, TNF-α (or IL-4) and TGF-β (Ito et al. 1999).

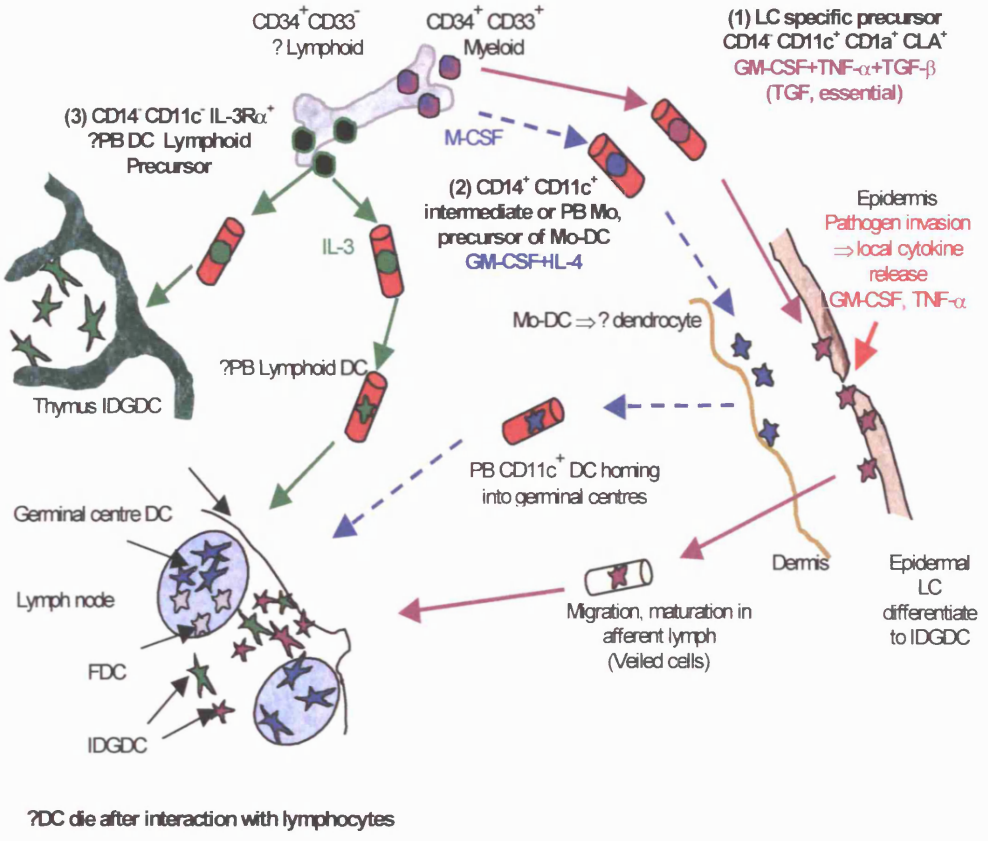
Commitment to LC development is made at the $CD34^+$ stage (Maurer and Stingl 1999). $CD34^+$ cells expressing the cutaneous leucocyte associated antigen (CLA) differentiate into LCs while the $CD34^+CLA^-$ precursors develop into interstitial type DC (INTDC) (Caux et al. 1999) via $CD14^+CD1a^-$ precursors (Strunk et al. 1997). Although Mo-DC express CD1a they lack LC features (like Birbeck granules and the Lag antigen) and are more related to the *in vivo* INTDC (e.g. dermal DC, also known as dendrocytes), which migrate to secondary lymphoid organs via PB. There they differentiate as $CD11c^+$ GCDC (Grouard et al. 1996). Mo-DC also have other features of dermal DC, like expression of CD68 and the coagulation factor XIIIa, (Caux et al. 1999). Myeloid DC may also develop from $CD15^+$ granulocyte precursors (Oehler et al. 1998).

(ii) Lymphoid DC: It is thought that these are derived from a $CD33^-$ lymphoid progenitor whose existence has not been conclusively proven (Galy et al. 1995; Res et al. 1996). The common lymphoid progenitor is thought to give rise to a Lin^-CD11c^- IL-$3R\alpha^+$ precursor DC (Grouard et al. 1997) that was initially described as a plasmacytoid T cell (Grouard et al. 1997) or a plasmacytoid monocyte (Cella et al. 1999; Palucka and Banchereau 1999; Siegal et al. 1999). Lin^-CD11c^-IL-$3R\alpha^+$ cells express low levels of GM-CSFRα and high levels of IL-3Rα consistent with their *in*

vitro cytokine requirements (Banchereau et al. 2000; Brossart et al. 2001). The progeny of these cells lacks myeloid markers (e.g. CD33) (Brossart et al. 2001) and is thought to correspond to the $CD11c^-$ DC found in the thymus, T cell areas of lymphoid organs and PB (Young 1999a). In mice, lymphoid DC have been characterized (Ardavin et al. 1993; Saunders et al. 1996; Vremec et al. 1992; Wu et al. 1996). They are derived from lymphoid-committed precursors, which have the potential to develop into B, NK, T cells and DC but not myeloid lineage cells (Ardavin et al. 1993; Wu et al. 1996). These early thymic precursors generate thymic DC *in vitro* and do not require GM-CSF (Saunders et al. 1996). Murine lymphoid-derived DC express CD8α which is also expressed by murine splenic and lymph node DC (Vremec and Shortman 1997). A recent study however has shown that CD8α is not a marker of lymphoid origin in mice (Traver et al. 2000). Galy and co-workers (Galy et al. 1995) identified a human BM lymphoid progenitor that has the potential to differentiate to all types of lymphocytes (T, B, NK cells) as well as DC. This $CD34^+Lin^-CD10^+$ progenitor has no myeloid differentiation potential and does not survive in the presence of cytokines that promote monocyte differentiation (Galy et al. 2000).

Lymphocytes and DC share cell surface markers such as CD1, CD2, CD4 and CD8 in humans and mice (Sotzik et al. 1994; Takimizawa et al. 1997; Vremec et al. 1992; Wu et al. 1991). More importantly DC appear to be the last cell to develop prior to T-cell differentiation (Galy et al. 1995) thus demonstrating that they are more closely related to T cells than B/ NK cells. It is noteworthy that over expression of the Ikaros-7 protein (Ik-7), a vital protein essential for normal lymphopoiesis, in human BM $CD34^+$ cells, inhibited the development of lymphoid DC but not that of Mo-DC (Galy et al. 2000).

Fig 1.3b The Life Cycle of DC. Adapted from (Banchereau et al. 2000; Caux et al. 1999).



A common myeloid progenitor can develop into LC (1) or monocytes (2), which in turn may differentiate into Mo-DC. Mo-DC may also develop directly from $CD34^+$ cells via the $CD14^+CD11c^+$ bipotential intermediate. (3) A lymphoid progenitor may give rise to PB $CD14^-CD11c^-IL\text{-}3R\alpha^+$ lymphoid DC. CLA, cutaneous leucocyte homing antigen; LCs, Langerhan's cells; Mo-DC, monocyte-derived DC; FDC, follicular DC; IDGDC, interdigitating DC.

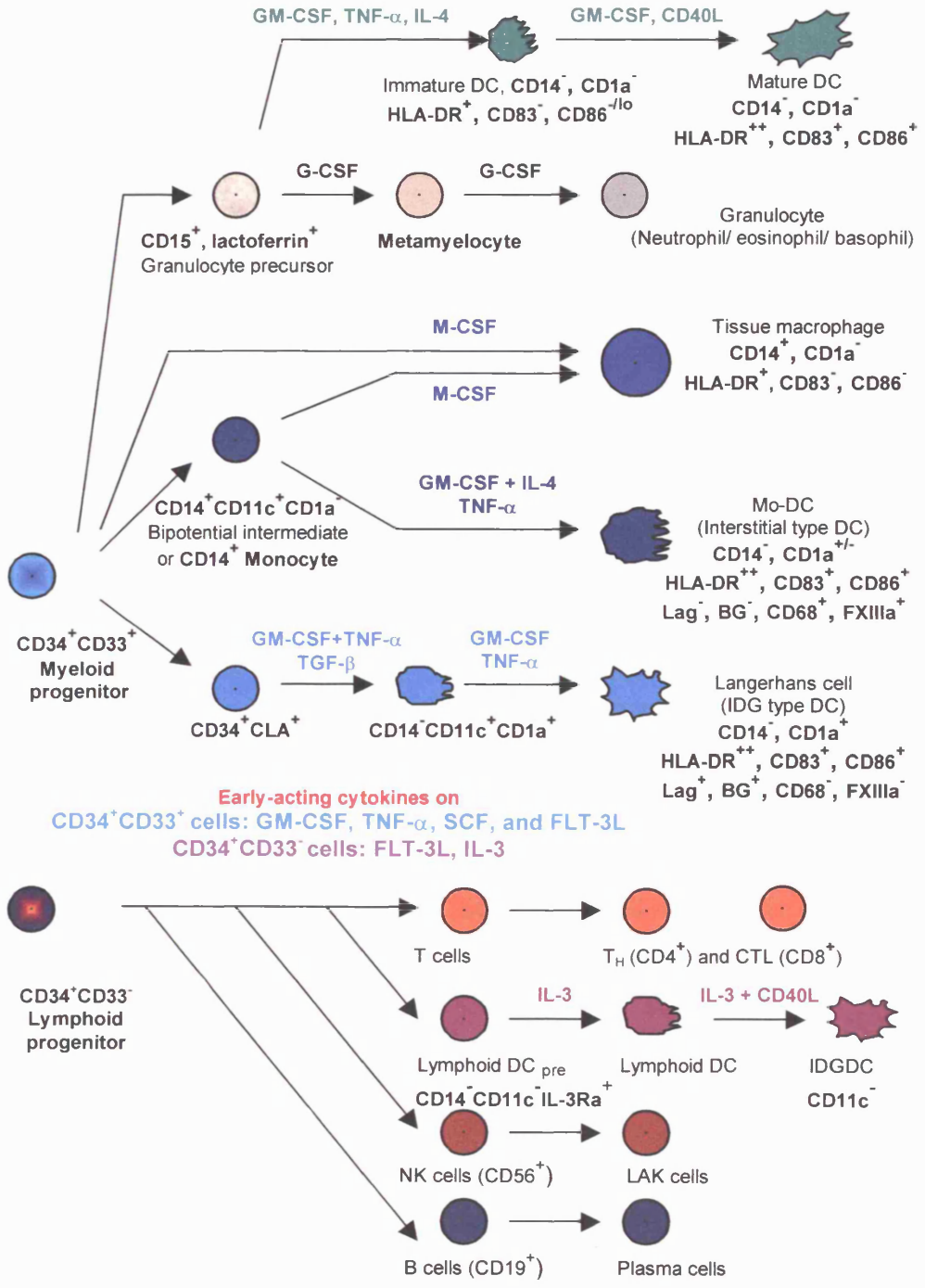
Table 1.3b Myeloid and Lymphoid DC- Phenotypic and Functional Differences.

Adapted from (Young 1999a).

	Myeloid DC	Myeloid DC	Lymphoid DC
Cell type generated *in vitro*			
	Blood Mo-DC† or $CD34^+$ derived DC (via $CD14^+$ intermediate)	$CD34^+$ derived DC (no $CD14^+$ intermediate)	$CD11c^-$, $CD33^-$ Precursor (Mature form not well defined in human)
Cell type ***in vivo*** **(Peripheral tissues)**			
	Interstitial DC (includes dermal DC)	Langerhans cells	Not defined in human
Cell type ***in vivo*** **(Lymphoid tissues)**			
	Germinal centre DC	IDGDC†	IDGDC†, thymus or Para follicular T-cell zones (Both $CD11c^-$)
Progenitor phenotype	$CD14^+CD11c^+CD1a^-$	$CD14^-CD11c^+CD1a^+$	$CD14^-CD11c^-$ IL-R3α^+
Cytokine requirements	GM-CSF plus IL-4 or TNF-α	GM-CSF + TNF-α TGF-β essential for LC	IL-3, CD40L, (GM-CSF independent)
Function	Ag presentation to memory T cells (B cell activation, only interstitial DC)	Ag presentation to and activation of naïve and memory T cells	Central (thymus) and peripheral tolerance (Lymph nodes)

† Mo-DC, monocyte-derived DC; IDGDC, Interdigitating DC.

Fig 1.3c Myeloid and Lymphoid DC Development.



Adapted and modified from (Caux et al. 1999; Maraskovsky et al. 1999; Young 1999a; Young 1999b). BG, Birbeck granules; Lag, Langerhans cell antigen; FXIIIa, factor 13a; IDGDC, Interdigitating DC; CTL, Cytotoxic T lymphocytes; NK, Natural killer cells; LAK, lymphokine-activated killer cells.

1.3.4 Cytokine requirements for human DC development

Although DC have the capacity to produce a variety of cytokines (Zhou and Tedder 1995b) they require exogenous growth factors for expansion and differentiation from their precursor cells. The two most important and essential cytokines for the generation of myeloid DC are GM-CSF + TNF-α if using $CD34^+$ HPC (Caux et al. 1992; Caux et al. 1999) and GM-CSF + IL-4 if using PB monocytes (Romani et al. 1994; Sallusto and Lanzavecchia 1994; Zhou and Tedder 1996). IL-4 blocks the differentiation of monocytes (Bender et al. 1996; Jansen et al. 1989; Romani et al. 1994; Romani et al. 1996; Sallusto and Lanzavecchia 1994) however it can be also used with GM-CSF + TNF-α in the generation of DC from $CD34^+$ HPCs (Young 1999a). Mo-DC however require additional maturation *in vitro* and for this Mo-CM, TNF-α or CD40L can be used (Banchereau et al. 2000; Fong and Engleman 2000). In the generation of myeloid DC IL-3 can be substituted for GM-CSF (Caux et al. 1996b) and IL-13 for IL-4 (Piemonti et al. 1995; Zurawski and de Vries 1994). TGF-β is essential for LC generation *in vivo* and *in vitro* –in serum-free cultures- (Maurer and Stingl 1999). The early acting HGFs, SCF and FLT-3L enhance the expansion of progenitors rather than differentiation (Santiago-Schwarz et al. 1995; Siena et al. 1995; Szabolcs et al. 1995; Young et al. 1995). Less is known about the growth factor requirements of lymphoid DC. GM-CSF is not required (Saunders et al. 1996). IL-3 is essential for their survival and CD40 ligand (CD40L) for maturation (Grouard et al. 1997; Strobl et al. 1998). FLT-3L expands lymphoid as well as myeloid DC (Maraskovsky et al. 1996; Maraskovsky et al. 1999).

1.3.5 Dendritic cells and immunity

DC are key players in the control of immunity because of their ability to stimulate T and B cells (Fig 1.3d). DC are distributed at sites, which allow them to capture foreign antigen (Ag). DC migrate rapidly to sites of Ag deposition (within an hour) (McWilliam et al. 1996; McWilliam et al. 1994). This is mediated by production of chemokines at sites of inflammation (Banchereau et al. 2000) which chemoattract DC that bear chemokine receptors. Immature DC take up foreign microbial Ag in a number of ways: phagocytosis (Albert et al. 1998; Inaba et al. 1993; Moll 1993; Reis e Sousa et al. 1993), macropinocytosis (i.e. uptake of extracellular fluid/ solutes by pinocytic vesicles) and adsorptive (receptor-mediated) endocytosis (Engering et al. 1997; Fanger et al. 1996; Jiang et al. 1995; Mommaas et al. 1999; Sallusto et al. 1995). The captured Ag is directed to lysozome-related intracellular compartments rich in MHC-class II molecules (MHC-class II rich compartments or MIICs) where peptides are loaded to MHC class II molecules. Maturation/ inflammatory signals promote translocation of the MHC II-peptide complexes to the cell surface for recognition by $CD4^+$ T cells (reviewed in (Banchereau et al. 2000)). The maturation of DC induced by stimuli (e.g. TNF-α) following Ag capture is characterized by phenotypic and functional changes (Fig 1.3e). These involve changes in chemokine responsiveness, enhanced motility and migration to regional lymph nodes via afferent lymphatics ("veiled cells"). Additionally there is upregulation of co-stimulatory and cell adhesion molecules all of which facilitate interaction with and activation of naive (or memory) T cells. Processed Ag presented within MHC class II molecules results in the activation of $CD4^+$ T helper cells. Processed Ag is also presented within MHC class I molecules to $CD8^+$ cytotoxic cells (CTLs), which if activated proliferate (reviewed in (Banchereau and Steinman 1998)).

Mature DC produce IL-12 as a result of the DC-T cell interaction (Cella et al. 1996; Koch et al. 1996). IL-12 facilitates a T helper 1 (Th1) immune response in which $CD4^+$ T helper cells, produce IFN-γ. IFN-γ activates macrophages and combined with IL-12 enhances the differentiation of T cells into $CD8^+$ cytotoxic cells (CTLs) (Young 1999a). However in the presence of IL-4, DC promote the differentiation of T cells into Th2 cells, which secrete IL-4 and IL-5; these promote Ig production and eosinophil activation (reviewed in (Banchereau and Steinman 1998)). In this way DC stimulate Ag-specific primary (or secondary) immune responses. The activated T cells proliferate and travel to the initial site of Ag deposition/ inflammation. B cells are activated after contact with T cells and DC. B blasts migrate to other areas of lymphoid tissue (e.g. medulla of lymph nodes) and some of them become plasma cells producing Ig. This antibody-mediated immune response helps neutralize the invading microbe. DC are thought to die or be destroyed by the efferent immune response.

The type of DC involved influences the type of immune response generated. For example lymphoid-derived DC from $CD14^-CD11c^-IL\text{-}3R\alpha^+$ precursors direct $CD4^+$ T cells towards a Th2 cytokine response, i.e. one characterized by IL-4, IL-5, IL-10 and Ig production. Hence the DC involved in generating this are referred to as type 2 DC and their precursors as pDC2 (Brossart et al. 2001). Mo-DC are referred to as type 1 DC because they promote Th1 differentiation (Rissoan et al. 1999). Recent work has shown that pDC2 are in fact the "natural IFN-producing cells" (IPCs). IPC are the main leucocytes producing IFN-α, in response to microbes or tumour cells (Siegal et al. 1999). DC2 precursors are found in human tonsil and PB, have plasmacytoid morphology, are Lin^-CD4^+MHC class II^+ and differentiate to DC2s after culture with IL-3 and CD40L (Siegal et al. 1999). IPCs produce 200-1,000 times more IFN than other PB cells (Siegal et al. 1999) and when they mature they activate T helper cells

to promote Ig production. This provides a useful link between innate and adaptive immune responses (Banchereau et al. 2000; Hagmann 1999a).

DC maturation is promoted by factors, such as, whole bacteria, the bacterial-cell wall lipopolysaccharide (LPS), pro-inflammatory cytokines (IL-1, TNF-α), PGE-2, IFN-α or β, GM-CSF, T cells –mediated through CD40L- and viral RNA (Caux et al. 1999; Maraskovsky et al. 1999; Young 1999a; Young 1999b). Mature DC are poor at Ag uptake but they have very high levels of MHC class I and II molecules, costimulatory and cell adhesion molecules. The phenotypic differences of immature and mature DC are summarized in Fig 1.3e. DC are also involved in induction of tolerance; that is they tolerize T cells to self-antigen and prevent an attack on the body's own organs and tissues (Banchereau and Steinman 1998).

Fig 1.3d The Afferent and Efferent Arms of the Immune Response. Adapted from (Banchereau and Steinman 1998).

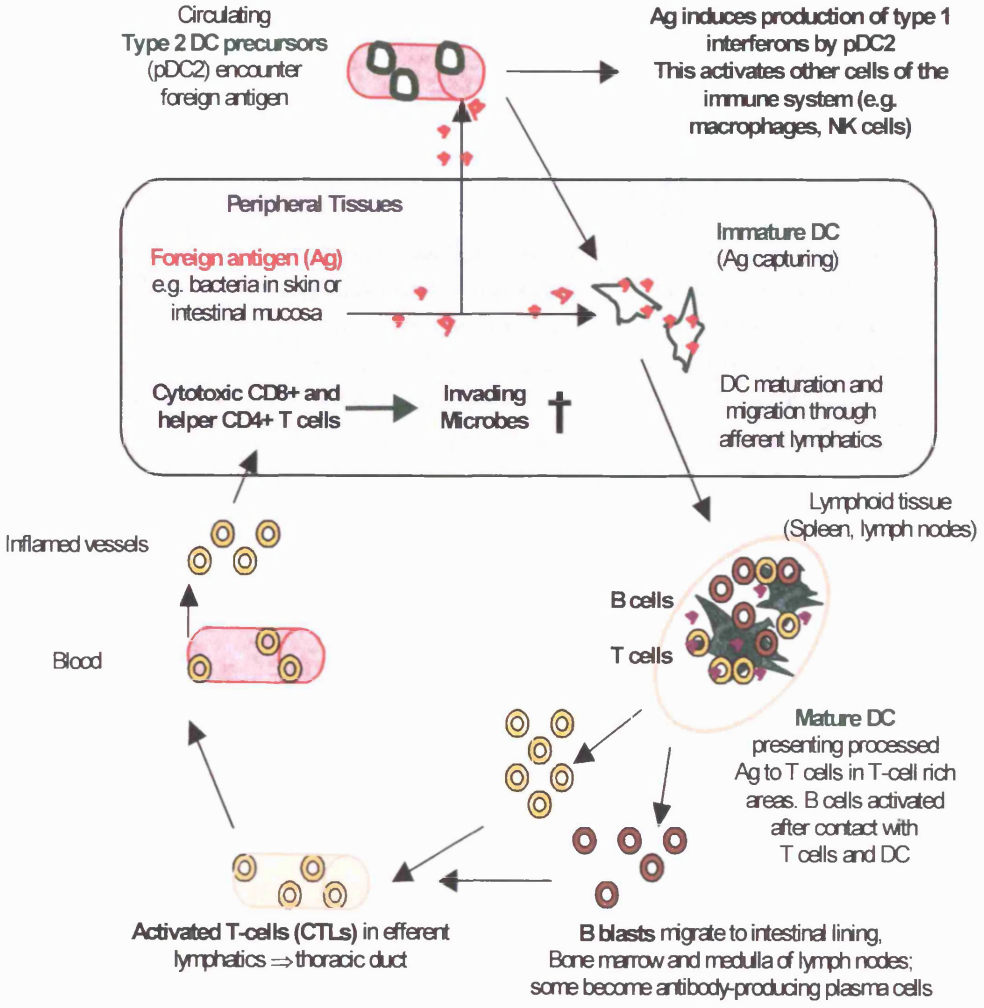
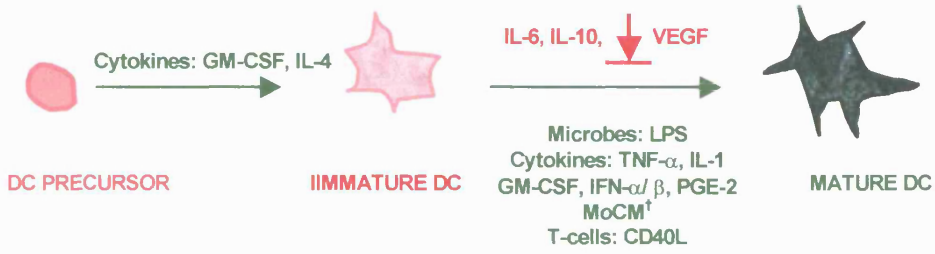


Fig 1.3e Differences between Immature and Mature DC (Banchereau and Steinman 1998; Hagmann 1999a; Young 1999a).



Production of **IFN-alpha** TNF Il-1	High intracellular MHC II (MIICs) **High endocytosis/ phagocytosis** Low cell adhesion molecules (CD11, CD50, CD54, CD58) Low IL-2Rα (CD25), IL-12 Low costimulatory molecules (CD40, CD80, CD86) Low CD83, p55 Low Chemokine receptors	**High surface MHC class I and II** Low endocytosis and Fc receptors High cell adhesion molecules (CD11, CD50, CD54, CD58) High IL-2Rα, IL12 **High costimulatory molecules** (CD40, CD80, CD86) High CD83, p55 High Chemokine receptors Highly motile, chemoattract T cells

† Mo-CM, monocyte-conditioned medium; PGE-2, prostaglandin E-2; CD40L, CD40 ligand; IL-2Rα, interleukin-2 receptor alpha; Red arrow and horizontal line illustrate the inhibitory effects of IL-6, IL-10 and VEGF.

1.3.6 Interactions of DC with other cells

DC-T cell interaction

This has essentially two functions. The priming of naive T cells in order to initiate primary immune responses and stimulation of effector T cells for secondary immune responses. The interaction between the T cell receptor (TCR) and the antigenic peptide-MHC complexes is dynamic (Fig 1.3f). T cells sense stimulation by DC ("signal 1") and are activated above a threshold level (Viola and Lanzavecchia 1996). This threshold level is tunable by costimulation ("signal 2") and explains why mature DC with higher levels of costimulatory molecules (CD80/ CD86) are more efficient at naive T-cell priming than immature DC. Stimulation of DC via ligation of CD40 (by CD40L, expressed by T cells) is vital in enhancing the T cell response (Caux et al. 1994; Cella et al. 1996), expression of CD80/ 86 and cytokine production by the DC (Bennett et al. 1998; Sallusto and Lanzavecchia 1994; Schoenberger et al. 1998).

Different levels of TCR engagement produce different responses (Fig 1.3g). Proliferation and cytokine production need higher levels of TCR occupancy than cytotoxicity (Valitutti et al. 1996). The threshold for TCR triggering is decreased by costimulatory signals delivered by CD28 (expressed on T cells) to its cognate receptors (CD80 and CD86) found on DC. The DC-T cell interaction is further stabilized by intercellular adhesion molecules (ICAMs), which enable the signal to be sustained for hours. The fate of T-cells depends on the presence of co-stimulatory signals and their total duration. Effector T-cells are less demanding than naive T-cells and can interact with non-professional APCs. Naive T cells need a minimum of 10 hours of sustained signalling with costimulation and up to 30 hours without for activation (Iezzi et al. 1998). Non-professional APCs that interact transiently with naive T cells cannot activate them and are likely to induce anergy/ tolerance. Effector

T cells however can be activated by APCs or by non-professional APCs very quickly (Viola et al. 1999).

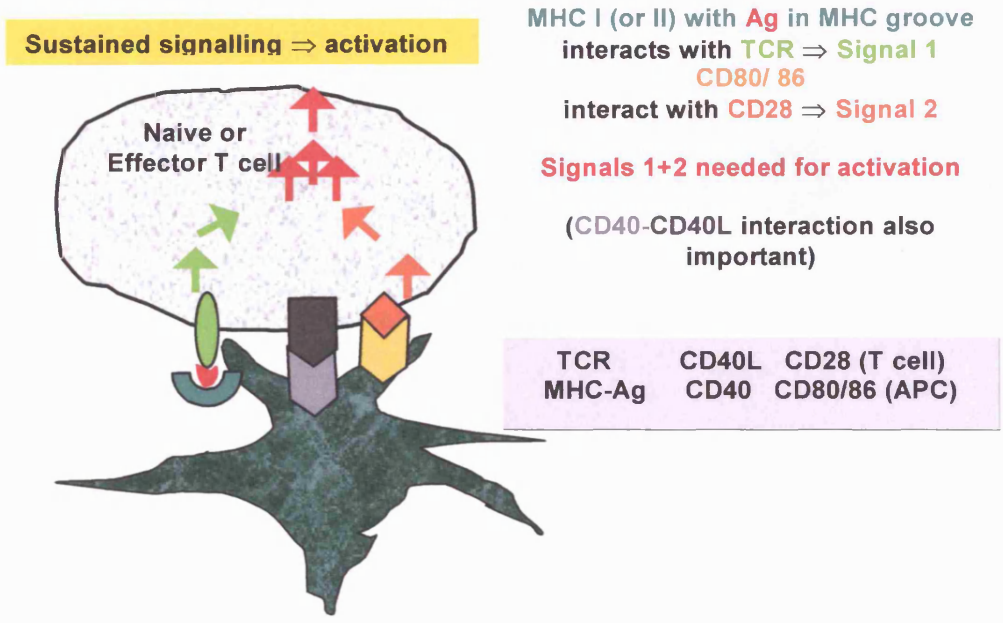
DC-NK cell interaction

Natural Killer cells are BM-derived lymphocytes, which kill certain tumour cell lines without prior sensitization (Kiessling et al. 1975). They have the phenotype of large granular lymphocytes, are $CD3^{-}CD56^{+}$ and lack Ig expression (Chambers and Ljunggren 1999). They play an important role in the innate immune response against viruses, intracellular bacteria and parasites (Bancroft 1993; Biron 1997; Scharton-Kersten and Sher 1997; Scott and Trinchieri 1995). DC and NK cells in humans share some features such as a common lymphoid progenitor with B and T cells (Galy et al. 1995). NK cells secrete cytokines like IFN-γ, GM-CSF, and TNF-α and can therefore fight infections early on, like DC, which rapidly migrate to sites of inflammation (McWilliam et al. 1996). Spleen DC have shown NK cell-like cytotoxicity *in vitro* (Josien et al. 1997) and MHC class II^{+} NK cells have shown DC-like activity, by stimulating allogeneic T-cells *in vitro* (Scala et al. 1985). The significance of the *in vivo* interactions between DC and NK cells (Fig 1.3h) however is uncertain.

DC-B cell interaction

DC have major effects on B cell growth and secretion of Igs (Banchereau and Steinman 1998). INTDC promote the differentiation of CD40L-activated naive B cells to plasma cells (Dubois et al. 1997). DC are found in mucosal–associated lymphoid tissue (Frankel et al. 1996; Kelsall and Strober 1997) and are important in mucosal immunity. They induce Ig class switch (from IgM to IgA) on naive B cells activated by CD40L from T cells (Fayette et al. 1997). This requires IL-10 and TGF-β (Banchereau et al. 2000). DC also help CD40-activated memory B cells differentiate to IgG-secreting cells (Dubois et al. 1998; Dubois et al. 1997).

Fig 1.3f Interaction between DC and T cells. Adapted and modified from (Viola et al. 1999).



For activation naive T cells need >20 hours interaction but effector cells just 1 hour (Viola et al. 1999).

Fig 1.3g Outcome of DC-T Cell Interaction. Adapted from (Viola et al. 1999).

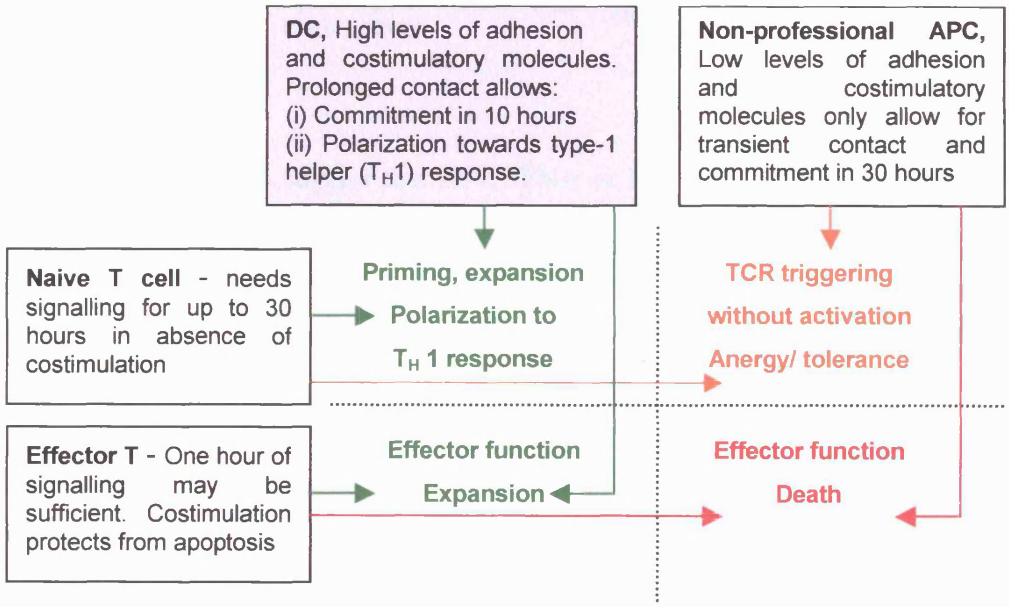
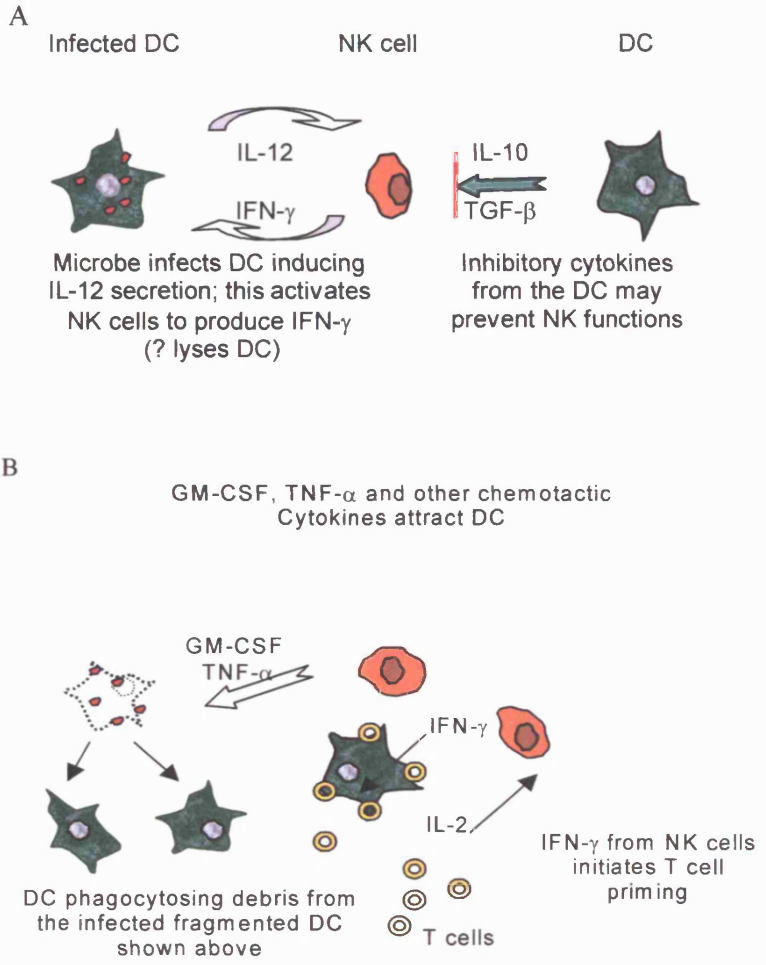


Fig 1.3h Hypothetical Interactions between DC and NK cells *in vivo*. Adapted and modified from (Chambers and Ljunggren 1999).



A. *In vitro* animal data suggest that IL-2, IFN-γ or IL-2/ IL-12 stimulated NK cells can lyse murine DC. Cytokines like IL-10 and TGF-β secreted by DC may inhibit NK cell function. B. GM-CSF and TNF-α from NK cells may promote DC maturation *in vivo* while IFN-γ may facilitate T-cell priming.

1.3.7 DC and chemokines

Chemokines are a large family of cytokines involved in the movement and production of blood cells (reviewed by (Maekawa and Ishii 2000)). To date more than 50 chemokines have been identified which are divided broadly into 4 families, based on the position of the conserved cysteine (C) residue near the amino terminal part of the molecule. Chemokines exert their effects through specific receptors. Immature DC produce inflammatory chemokines (e.g. MIP-1α) and express chemokines receptors, which allow them to migrate to sites where they are required (Caux et al. 1999). For example the MIP-3α receptor, CCR6, directs immature DC to sites of inflammation where MIP-3α is produced. Upon DC maturation responsiveness to MIP-3α declines and DC upregulate CCR7 that helps direct DC to lymphoid tissue where MIP-3β is produced (reviewed in (Banchereau et al. 2000; Brossart et al. 2001; Young 1999a)). In the T cell areas of the lymph node DC produce DC-CK1 (Adema et al. 1997) and MDC (Godiska et al. 1997) (macrophage-derived chemokine) chemokines, which chemoattract naive and memory T cells.

1.3.8 Clinical importance of dendritic cells

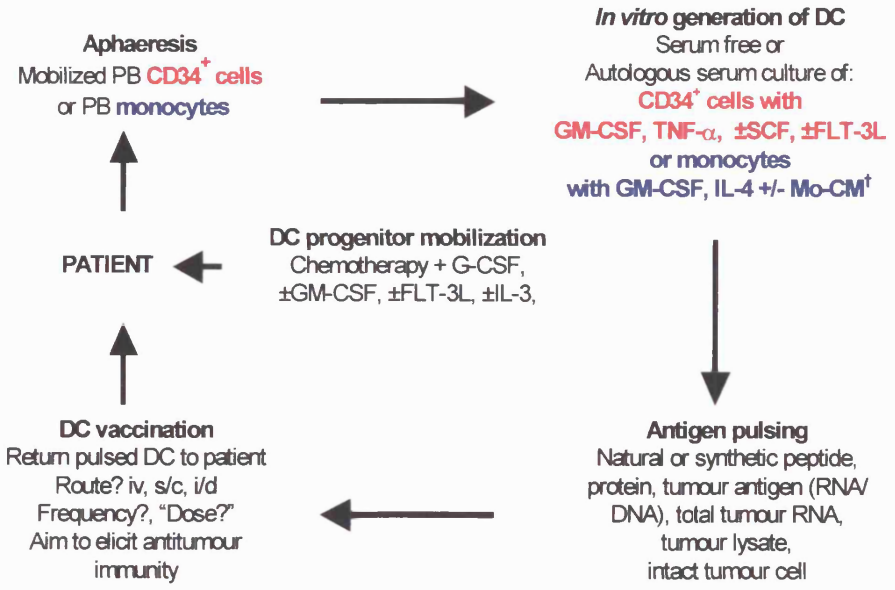
From the preceding sections it is clear that DC are crucially important for the normal physiological function of the immune system by controlling T, B and NK cell function in health (Banchereau and Steinman 1998; Steinman 1991) and also in disease states such as cancer, infections (including HIV-1), organ rejection, autoimmunity, allergy (including asthma) as well as graft-versus host disease (GVHD) (Hart 1997; Steinman 1996; Stingl and Bergstresser 1995; Young 1999a). From the point of view of therapeutics there is a huge potential for the use of DC-based therapies (Gluckman et al. 1997; Greten and Jaffee 1999; Mayordomo et al.

1997; Steinman 1996; Timmerman and Levy 1999) to treat these diseases in humans and animals. For humans this involves the application of novel modalities of treatment in the fight against cancer using DC based vaccination (Brossart et al. 2001; Greten and Jaffee 1999; Mayordomo et al. 1997; Pardoll 1998; Steinman 1996; Timmerman and Levy 1999). The principle behind this approach relies on the antigen processing/ presentation capability of DC. Autologous DC co-cultured (pulsed) with tumour specific or tumour associated antigens can then be used to stimulate cytotoxic T cell responses in tumour bearing hosts (Fig 1.3i).

Another approach has been the use of fusion vaccines. In a recent clinical study Kugler and co-workers (Kugler et al. 2000) used fusion vaccines consisting of autologous tumour cells - allogeneic DC in advanced metastatic renal cell cancer and achieved a remarkable response rate of 41%. This is very encouraging in a disease with a response rate to systemic treatment of 10% (Kufe 2000). In addition to cancer, experimental DC vaccines against HIV-1 (Lotze and Jaffe 1999) have been used in clinical trials. Tolerogenic DC have been used in mice for the treatment of allograft rejection (Lu et al. 1999). Finally work in mice has also demonstrated that manipulation of host APCs can be used to overcome a commonly encountered complication of BM transplantation, GVHD (Hagmann 1999b).

The central role of DC in the initiation of immune responses led to extensive investigation of their potential for vaccination (Timmerman and Levy 1999). DC are ideal targets because they express 50-fold higher levels of MHC molecules than macrophages and so they provide more MHC-peptide complexes for T-cell receptor ligation (Pardoll 1998). They express very high levels of adhesion and costimulatory molecules (Pardoll 1998) and have genes encoding for T-cell specific chemokines like DC-CK1 (Adema et al. 1997).

Fig 1.3i The Basic Principle of Dendritic Cell-based Immunotherapy. Adapted from (Morse and Lyerly 1998; Steinman 1996).



Dendritic cells are obtained by serum-free *ex-vivo* culture of either $CD34^+$ progenitors or PB monocytes using different cytokine combinations. DC are then co-cultured with appropriate antigen/s derived from the patient's tumour. † MoCM, monocyte-conditioned medium.

1.3.9 DC and cancer

The presence of higher numbers of DC within different types of solid tumours is associated with a better prognosis (Lotze and Jaffe 1999). Most studies, which were done in the early 1990's, have shown a correlation between the degree of DC infiltration and improved prognosis for solid tumours (see table 1.3c). DC are thought to be the link between innate and adaptive immunity against tumours (Banchereau et al. 2000). Precursors of DC2 produce IFN-α which has antitumour activity while at other stages of differentiation, DC control the activity of T and B cells and possibly NK cells (reviewed in (Banchereau et al. 2000; Brossart et al. 2001; Sprinzl et al. 2001)). There is also evidence from murine models that DC cause apoptosis in some tumours (Lotze and Jaffe 1999). The principal effectors of antitumour immunity are the $CD8^+$ CTLs (Roth et al. 1994) hence DC are ideal means to use for CTL expansion. DC can polarize the immune response towards Th1 or Th2 type and provide plasticity as their phenotype/ function is altered by micro environmental signals (Banchereau et al. 2000). They are therefore potential targets for generating strong antitumour immune responses by recruiting all available effector cells (Sprinzl et al. 2001).

Tumours employ several mechanisms to evade immune surveillance. For example direct infiltration of BM/ lymphoid tissues, inhibition of (fully functional) DC generation (Gabrilovich et al. 1996b; Gabrilovich et al. 1996a) and altered or reduced expression of MHC molecules (Pardoll 2000). Some tumours produce immunosuppressive factors like IL-6, IL-10, VEGF and M-CSF, which inhibit DC maturation/ differentiation *in vitro* (Banchereau et al. 2000; Gabrilovich et al. 1996c).

Table 1.3c Relationship between DC Infiltration and Prognosis in Solid Tumours. Modified from (Lotze and Jaffe 1999).

Tumour Group	DC Infiltration/ Prognosis
Lung	Improved prognosis
Breast	? Improved prognosis
Prostate	Improved prognosis
Gastric	Improved prognosis
Oesophageal	Improved prognosis
Hodgkin's disease	FDC improve prognosis
Basal cell carcinoma	Less in tumours
Melanoma	Inverse with tumour thickness
Mycosis fungoides	Markedly improved prognosis
Skin tumours	Reduced DC infiltration
Head and neck	Improved prognosis
Cervix	Improved prognosis
Endometrial	LC infiltration favourable
Thyroid	No effect

The use of DC to treat cancer has developed during the past 5 years as a result of a better understanding of tumour immunology and significant advances in the biology and generation of DC (Timmerman and Levy 1999).

Preclinical studies: Work in animal tumour model systems showed that *ex vivo* delivery of purified tumour antigen to a specific population of APC (DC enriched preparations) conferred antitumour immunity (reviewed in (Timmerman and Levy 1999)). With the development of techniques which allowed the *in vitro* generation of large numbers of DC, it has been shown that BM derived DC pulsed with class I restricted peptides (derived from tumour antigens) induced antitumour CTL responses and protected vaccinated mice against tumours (Celluzzi et al. 1996; Mayordomo et al. 1995; Porgador et al. 1996).

Clinical studies: Early clinical trials have established the feasibility and safety of DC vaccination and have demonstrated its activity in several tumour types (Timmerman and Levy 1999). The first reported clinical trial of DC vaccination used idiotype-pulsed DC from patients with chemotherapy-resistant low-grade non-Hodgkin's lymphoma (see chapter 5) (Hsu et al. 1996). After the characterization of melanoma antigens several melanoma trials followed (Nestle et al. 1998; Panelli et al. 2000; Thurner et al. 1999). More recently the results of clinical trials in other tumour sites have been published; colorectal and lung cancer (Nair et al. 1999), renal cell carcinoma (Holtl et al. 1999; Kugler et al. 2000), prostate cancer (Murphy et al. 1999), ovarian and breast cancer (Brossart et al. 2000) and myeloma (Reichardt et al. 1999). Currently more than 1,000 patients worldwide are being treated in trials of DC vaccination (Salgaller et al. 1999). Table 1.3d summarises some of the important published and ongoing clinical trials of DC vaccination for cancer.

Table 1.3d Clinical Trials of DC Vaccination (Timmerman and Levy 1999).

Tumour†	Antigen‡	DC type§	Institution	Investigator
Bre, Co/re, Pan, Lun, Mel	Tumour cell lysate	Mo-DC (GM + IL-4)	Univ. Michigan	Mule (Fields et al. 1998)
Bre, Co/re, Lun	Mutant ras/ p53	Mo-DC (GM + IL-4)	Vanderbilt	Carbone
Bre, Co/re	CEA	Mo-DC (GM + IL-4)	Univ. South. California	Weber
Lun, Co/re	CEA	FLT-3L mobilized DC	Stanford Univ.	Fong, Engleman
Lun	CEA	Mo-DC (GM + IL-4)	Duke Univ.	Lyerly, Gilboa
Mel	gp100, MART-1, tyr	Mo-DC (GM + IL-4)	Univ. Zurich	Nestle (Nestle et al. 1998)
Mel	gp100, MART-1, tyr	Mo-DC (GM + IL-4) or $CD34^+$ cells	Univ. Pittsburgh	Lotze (Lotze et al. 1997)
Mel	gp100, MART-1, MAGE-3, tyr	$CD34^+$ cells (GM + TNF-α)	Baylor	Banchereau
Mel	gp100, tyr	Mo-DC (GM + IL-4)	Univ. South. California	Weber
Pros	PAP	Density purified DC	Stanford	Fong, Engleman
Pros	PSMA	Mo-DC (GM + IL-4) ± adj. GM-CSF	Northwest Biotherapeutics	Murphy (Salgaller et al. 1998)
Pros	PAP	Density purified DC	Dendreon	Valone (Valone et al. 1998)
Ren	Tumour cell lysate	Mo-DC (GM + IL-4)	UCLA	Figlin

† Bre, breast; Co/re, colorectal; Pan, pancreas; Lun, lung; Mel, melanoma; pros, prostate; ren, renal. ‡ CEA, carcinoembryonic antigen; gp, glycoprotein; tyr, tyrosinase; PAP, prostatic alkaline phosphatase; PSMA, prostate specific membrane antigen. § Monocyte-derived DC cultured in GM-CSF + IL-4 (GM+IL-4).

1.4 Characterization of blood stem cells

The last 40 years have seen enormous advances in the phenotyping of blood stem cells (Kvalheim and Smeland 1998). This is largely credited to the development of the following: (i) Monoclonal antibodies (MoAb) against leucocyte differentiation antigens (cluster of differentiation or CD antigens); (ii) assay systems for HPC; (iii) the identification and production by genetic engineering of numerous recombinant cytokines, which have allowed the study of their effects on haemopoietic cells.

1.4.1 The CD34 antigen

(i) Expression and function: CD34 is a transmembrane cell surface glycoprotein (Fig 1.4a) with molecular weight of 110-kDal. It was initially identified using the My10 MoAb (Civin et al. 1984). The CD34 antigen is uniquely expressed on human HPC, capillary endothelium and stromal cell precursors in BM (Andrews et al. 1986; Civin et al. 1984; Fina et al. 1990; Simmons and Torok Storb 1991; Smeland et al. 1992; Tindle et al. 1985). The $CD34^+$ cell population accounts for a small fraction of the total leucocytes (Table 1.4a) in BM, CB and PB (Fritsch et al. 1994; Kinniburgh and Russell 1993; Van Epps et al. 1994). The $CD34^+$ population includes, most pluripotent stem cells (Kvalheim and Smeland 1998) and most of the committed progenitor cells in humans (Graham and Wright 1997). Moreover enriched $CD34^+$ cells can reconstitute haemopoiesis *in vivo,* in humans and non-human primates (Berenson et al. 1988; Berenson et al. 1991). The function of the CD34 antigen however is still unknown although it is thought to be involved in cell adhesion (Holyoake et al. 1996) and signal transduction (Simmons et al. 1992).

Fig 1.4a The Structural Features of The Human CD34 and AC133 Antigens.

(Miraglia et al. 1997; Sutherland et al. 1993); (please see section 1.4.2 for AC133).

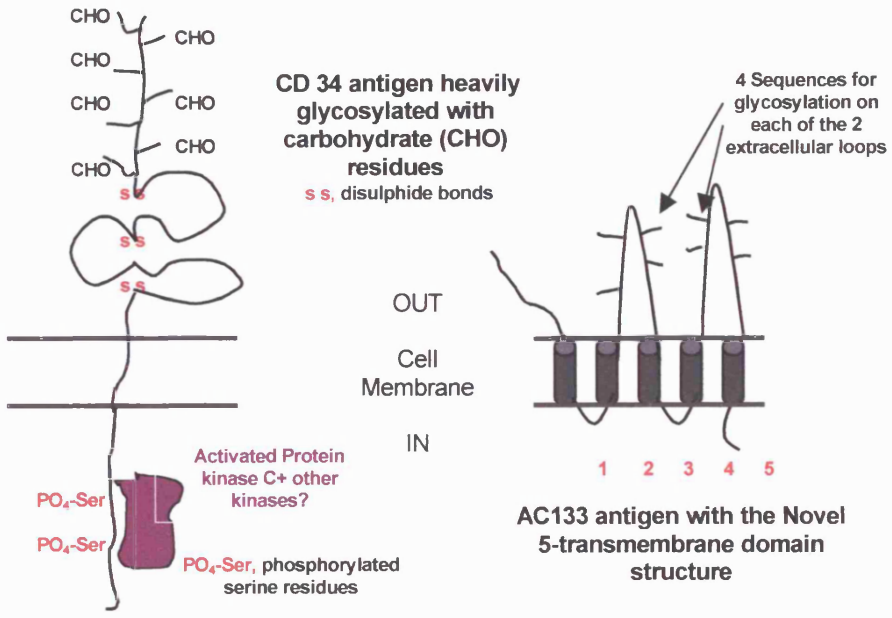


Table 1.4a Percentage of $CD34^+$ cells in BM, CB and PB at Steady State and after Mobilization.

Source of HPC	BM	CB	PB Steady state	PB Mobilized
CD34%	1-4%	1%	0.1%	1-4%
CD34% is expressed as a % of nucleated cells				
Reference	(Kvalheim and Smeland 1998)	(Fritsch et al. 1994; Kinniburgh and Russell 1993; Van Epps et al. 1994)	(Stadmauer et al. 1995)	(Kvalheim and Smeland 1998)

(ii) CD34 enumeration: The large increase in the number of autologous stem cell transplants using PBSC over the last decade has been accompanied by the adoption of more stringent quality controls for the autografts in use. Enumeration of $CD34^+$ cells has therefore become one of the 2 most important parameters used to assess the haemopoietic reconstitutive capacity of transplanted cells, the other being the CFC-GM assay (To et al. 1997). The current gold standard method to enumerate $CD34^+$ cells is that recommended by the International Society for Haematotherapy and Graft Engineering (ISHAGE) known as the ISHAGE guidelines (Sutherland et al. 1996). The details of this method are explained in chapter 2, however briefly, it involves the simultaneous use of CD34 and CD45 MoAb conjugated to fluorochromes. Using a combined gating strategy it is possible to measure the fraction of $CD34^+$ cells among the total leucocyte population.

Flow cytometry distinguishes 2 different populations of HPC. The cells, which strongly express CD34 ($CD34^{bright}$), include most of the immature progenitors. The cells expressing lower levels of CD34 ($CD34^{dim}$) include lineage-committed progenitors (Nieto and Shpall 1998). The CD34 antigen is expressed at high levels on the earliest HPC and its expression decreases as the cells differentiate and mature (Nieto and Shpall 1998; Watt and Visser 1992). Of even greater importance however is the 10-fold or more increase in the numbers of $CD34^+$ cells in PB after HPC mobilization (Kvalheim and Smeland 1998), with chemotherapy and growth factors. This is frequently used to determine the optimal time for aphaeresis (Fielding et al. 1994; Siena et al. 1993; Smith and Keating 1994; Stadmauer et al. 1995).

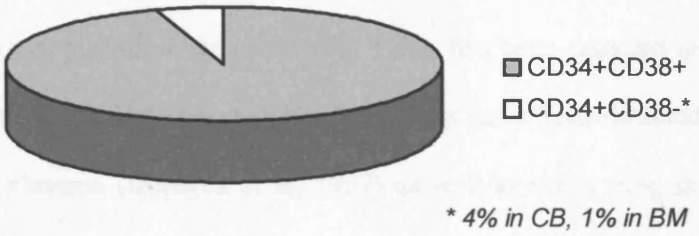
CD34 enumeration has advantages over the traditional clonogenic assay for CFC-GM. It is easy and quick to perform (takes ~1hour) and the results are available on the same day. This allows clinical decisions for a repeat or delayed aphaeresis, to be

made. In addition the CD34 assay enumerates all HPC not just those committed to the granulocyte/ macrophage lineage (To et al. 1997). Despite the fact multiple groups have tried to develop a reliable accurate CD34 assay by flow cytometry there is, as yet no universal consensus on a single method.

(iii) Subsets of CD34$^+$ cells: $CD34^+$ cells are heterogeneous (Graham and Wright 1997; Kvalheim and Smeland 1998) and contain most unipotent and multipotent committed progenitors (CFC-GM, CFC-DC, BFU-E, CFC-Meg etc) as well as $CD34^+CD38^-$ cells, which account for only a small percentage of the $CD34^+$ fraction (Fig 1.4b) and are more primitive. In turn true pluripotent stem cells are only a small fraction of the $CD34^+CD38^-$ population (~1/100,000 BM MNC) (Kvalheim and Smeland 1998). The most primitive cells are thought to be $CD34^+CD38^-$, $CD34^+HLADR^-$ and $CD34^+Thy\text{-}1^+$ (Craig et al. 1993; Huang and Terstappen 1994; Humeau et al. 1996; Rusten et al. 1994; Terstappen et al. 1991). $CD34^+CD38^-$ cells are thought to be more homogeneous and are enriched for primitive haemopoietic cells, detectable by *in vitro* assays, than $CD34^+HLA\text{-}DR^-$ cells (Huang and Terstappen 1994; Rusten et al. 1994).

Mobilized PB and CB $CD34^+$ cells contain relatively less B cell precursors and more cells co-expressing myeloid markers, than BM $CD34^+$ cells (Donahue et al. 1996; Inaba et al. 1994b; Van Epps et al. 1994). PB $CD34^+$ cells also express lower levels of adhesion molecules like leucocyte function antigen (LFA-1) and very late antigen-4 (VLA-4) than BM $CD34^+$cells (Mohle et al. 1993). The above suggest that $CD34^+$ cells from different sources show immunophenotype variations. These differences even include the proportion of actively cycling cells, which has been reported to be lower in $CD34^+$ cells from mobilized PB than BM (Donahue et al. 1996; Lemoli et al. 1997; Roberts and Metcalf 1995).

Fig 1.4b Heterogeneity of the CD34$^+$ Population. Adapted from (Kvalheim and Smeland 1998).



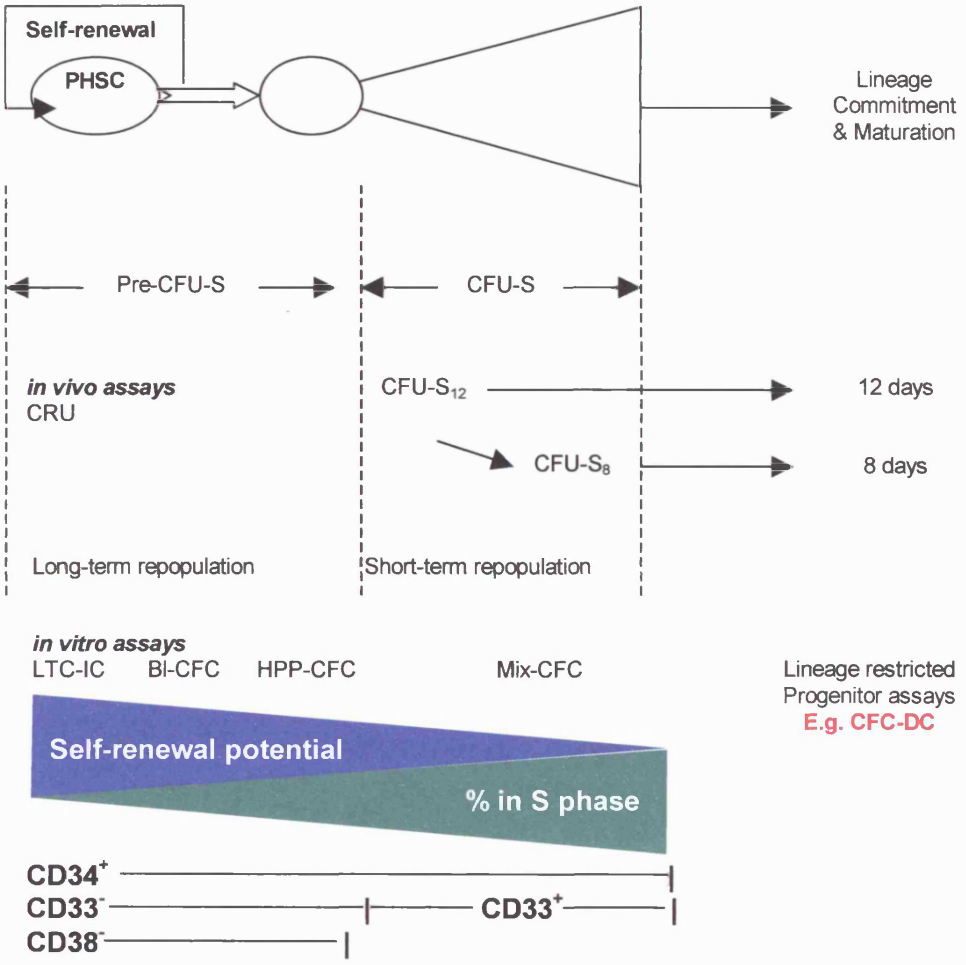
1.4.2 The AC133 antigen

AC133 is a monoclonal antibody that identifies a glycosylated protein with a molecular weight of 120-kDal expressed on $CD34^{bright}$ progenitors (Yin et al. 1997). The AC133 Ag, which has 5 transmembrane (5-TM) domains an extracellular N-terminus and a cytoplasmic C-terminus (Fig 1.4a), has been detected in BM only by immunohistochemistry; however m-RNA transcripts have been detected in pancreas, kidney, retina, placenta (Miraglia et al. 1997) as well as brain, lung skeletal muscle and testis (Miraglia et al. 1998). Its identification as an early marker of HPC is supported by its expression in some leukaemias and on granulo-monocytic and DC progenitors (de Wynter et al. 1998; Decatris et al. 1999; Yin et al. 1997). Its function is unknown however its presence on the surface of primitive undifferentiated cells suggests it could be a growth factor receptor (Miraglia et al. 1997).

1.4.3 Functional assays for HPC

Assays to characterize PBPC are based on functional characteristics of these cells, namely: commitment to haemopoietic lineage, potential to differentiate into more than one lineage and potential for self-renewal. For many years the cell that fulfilled these criteria was the spleen colony-forming unit (CFU-S) in the mouse (Till and McCulloch 1961). The basis of the CFU-S assay is the ability of transplanted HPC in the murine marrow to rescue potentially lethally irradiated recipient mice (Graham and Wright 1997). However work by Ploemacher and coworkers showed that marrow repopulating cells were outside the CFU-S compartment (Ploemacher and Brons 1988; Ploemacher and Brons 1989). Some of the most important HPC assays are described briefly below (see Fig 1.4c). This topic has been reviewed extensively in the literature (Graham and Wright 1997; Lord 1997; Watt and Visser 1992).

Fig 1.4c The Hierarchy of the Haemopoietic Stem Cell Populations and their Relationships with *in vivo* and *in vitro* Assays. Adapted and modified from (Graham and Wright 1997; Lord 1997; Moore 1991).



PHSC, primitive haemopoietic stem cell; CFU-S, spleen colony forming units; CRU, competitive repopulating unit; LTC-IC, long term culture-initiating cell; Bl-CFC, blast- colony forming cell (CFC); HPP-CFC, high proliferative potential-CFC. As pluripotent stem cells give rise to more mature progeny the capacity for self-renewal is lost and cells become more lineage restricted (i.e. committed to 1-3 lineages).

1.4.3 (i) In vivo assays for immature HPC

Marrow repopulating ability (MRA): MRA cells lie between primitive HSC and CFU-S in the stem cell hierarchy (Watt and Visser 1992). The MRA assay measures the ability of murine stem cells to repopulate the BM of lethally irradiated mice with new, day 12 CFU-S and CFC (Watt and Visser 1992).

Spleen colony forming units (CFU-S): *In vivo* assays were developed 41 years ago to enumerate murine stem cells. The CFU-S assay measures the capacity of mouse HPC to form macroscopically visible haemopoietic colonies in the spleens of lethally irradiated mice (Watt and Visser 1992). BM cells injected into such mice give rise to colonies on the spleen surface. These colonies are known as spleen colony forming units (CFU-S) (Till and McCulloch 1961). Moreover cells from these colonies were capable of forming new spleen colonies. CFU-S were initially identified as stem cells. CFU-S that appear early (day 8) lack stem cell properties (Moore 1991). CFU-S appearing later on day 12 are capable of self-renewal in serial transplant studies. It is now known that cellular fractions without CFU-S can restore haemopoiesis when transplanted into irradiated mice (Ploemacher and Brons 1988).

Xenografting approaches: The transplantation of human HPC into immune deficient mice has allowed us to study human stem cells in vivo. Currently it is unknown if these stem cells are identical to those repopulating human recipients.

SCID repopulating cell (SRC) assay: The *in vivo* study of human stem cells has been hindered by the absence of assays of their repopulating potential (Graham and Wright 1997). It was not until the late 1980's that the transplant of human HPC into sub lethally irradiated mice was attempted (Kamel-Reid and Dick 1988). Non-specific immune responses in recipient mice mean that large doses of cells are needed to overcome host resistance. This makes it difficult to do limiting dilution assays for

primitive repopulating cells. This has been overcome to some degree by the development of the non-obese diabetic (NOD) / severe combined immunodeficiency (SCID) mice. Such mice lack B/T lymphocytes because of the SCID defect and have impaired NK-cell, complement-mediated and macrophage function by virtue of the NOD defects (Graham and Wright 1997). Studies have shown that the human cells, which show multilineage engraftment in these mice, are $CD34^{+}CD38^{-}$. These cells, designated SCID repopulating cells or SRCs, are probably more primitive than the CFCs and the LTC-ICs –see below- (Larochelle et al. 1995; Nolta et al. 1996).

Fetal sheep repopulation assay system: This relatively recent development is based on the ability of human cells to engraft into fetal sheep (Zanjani 1997). The characteristics of these cells are very similar to the SRC (Dick 1999).

1.4.3 (ii) In vitro assays

In vitro assays are of two types, assays of HPC that require a preformed stroma for haemopoietic differentiation/ proliferation and quantitative clonogenic assays.

Assays needing a preformed stroma

Long-term culture-initiating cell assay (LTC-IC): The most primitive cells detectable by an *in vitro* assay require stromal support for growth. LTC-ICs are assayed *in vitro* by their ability to sustain haemopoiesis and progenitor cell generation in the presence of a preformed irradiated BM stroma (Sutherland et al. 1989). The assay involves culture of immature cells on a stromal cell layer for 5-8 weeks. These cells can then generate progenitors in standardized clonogenic assays. $CD34^{+}CD38^{-}$, $CD34^{+}Thy\text{-}1^{+}$ and $CD34^{+}HLA\text{-}DR^{-}$ cells are enriched in LTCIC although none of these populations contain all LTCIC (Prosper et al. 1996). Currently the LTCIC assay

is the best functional *in vitro* assay in humans for identifying primitive HPC (Sutherland et al. 1995; To et al. 1997).

Clonal assays: Bl-CFC, HPP-CFC and Mix-CFC

Blast-CFC (Bl-CFC): The Bl-CFC assay produces blast cells but does not promote their differentiation (Nakahata and Ogawa 1982). The assay detects early haemopoietic cells with high secondary recloning and self-renewal capacity capable of multilineage differentiation (Moore 1991).

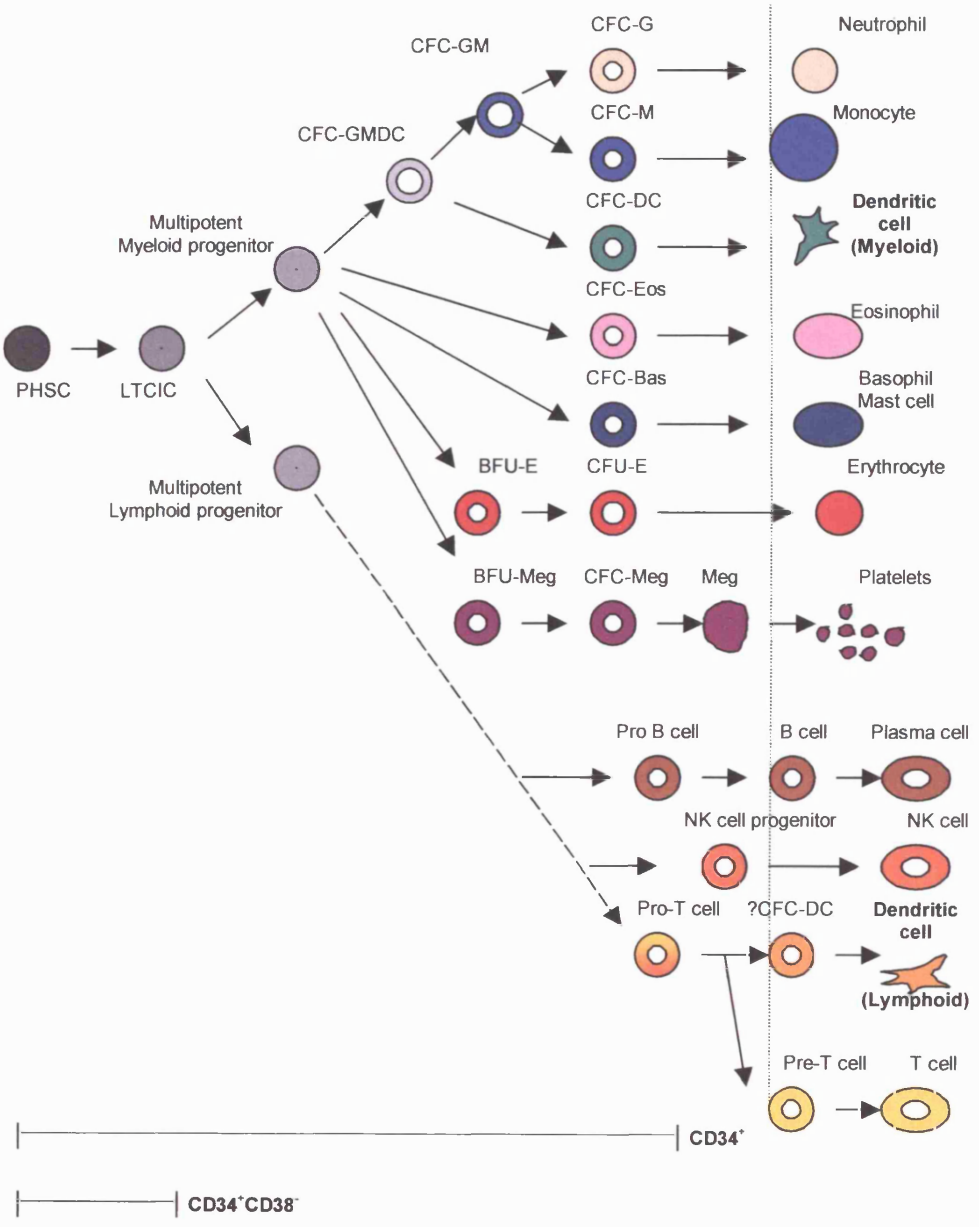
High proliferative potential (HPP-CFC): This is designed to detect cells with very high proliferative potential. The cells, which are resistant to the cytotoxic drug 5-fluorouracil (5-FU) are very primitive and generate macrophage colonies with 50,000 cells (Bradley and Hodgson 1979). The precise relationship with the Bl-CFC is unknown however the most primitive HPP-CFC may share properties with the Bl-CFC (Graham and Wright 1997).

Mix-CFC: The basic principle of this assay was based on the work of Bradley and Metcalf (Bradley and Metcalf 1966). It was then extended to cells with granulo-monocytic (Chervenick and Boggs 1971; McCredie et al. 1971), erythroid (Barr et al. 1975; Ogawa et al. 1977; Stephenson et al. 1971), megakaryocytic (Metcalf et al. 1975a) and lymphocytic potential (Metcalf et al. 1975b). Finally it was applied to cells with multi-lineage differentiation potential (Johnson and Metcalf 1977). Metcalf and colleagues first described this simple but ingenious assay (Johnson and Metcalf 1977), which was subsequently applied for human HPC (Ash et al. 1981; Fauser and Messner 1978; Fauser and Messner 1979). In essence it involves the culture of committed cells in semisolid medium, where the progenitors give rise to colonies of mature cells of at least 3 haemopoietic lineages. The clonogenic cell in this assay

generated progeny in which granulocytes (G), erythrocytes (E), macrophages (M) and megakaryocytes (M) were identified. Hence the assay name, CFC-GEMM. The frequency of the human CFC-GEMM is estimated to be $1/10^4$ normal BM MNC (Graham and Wright 1997). Under the influence of HGFs committed progenitors generate colonies consisting of cells of 1 or, more infrequently, 2-3 lineages (Kvalheim and Smeland 1998).

Clonal assays *in vitro* detect committed clonogenic progenitors. $CD34^+$ cells are cultured in semi-solid media and with appropriate cytokines the committed progenitors proliferate and differentiate to form colonies of mature cells. Each colony is derived from one progenitor (Fig 1.4d), which is designated by the prefix CFU (colony-forming unit) or CFC (colony-forming cell) followed by the cell type e.g. CFU-G (granulocyte), CFU-M (macrophage), CFC-DC, CFC-Eos (eosinophil), CFC-Bas, (basophil), CFC-Meg (megakaryocyte) and CFU-E (erythroid). Cells more primitive than CFU-E are designated as BFU-E (Burst-forming unit). The latter form large, often multicentric colonies, from which the smaller and less primitive erythroid colonies (CFU-E) are derived. The CFC-GEMM (or Mix) constitutes the base of the hierarchy in the "stem cell" compartment and overlaps to a large extent with the early (day 8) CFU-S.

Fig 1.4d Differentiation of CFC into Distinct Haemopoietic Lineages. Adapted and modified from (Heyworth et al. 1997; Kvalheim and Smeland 1998; Lyman and Jacobsen 1998).

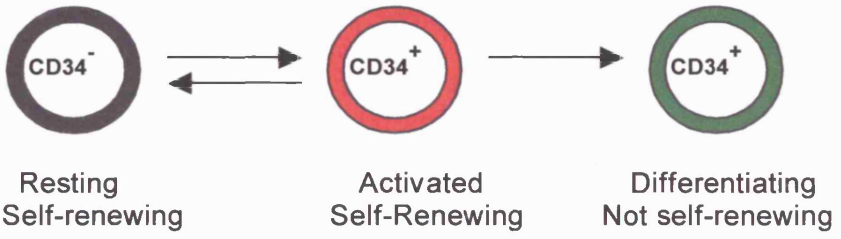


1.4.4 Current opinion on HSC

CD34 phenotype of human HPC

Since 1996 work has been published providing evidence for the presence of HSC in the CD34 negative ($^-$) fraction of BM cells. Osawa (Osawa et al. 1996) first showed that a single HSC that did not express the murine CD34 antigen could reconstitute haemopoiesis in a lethally irradiated mouse for nearly half the life span of the animal. Zanjani and colleagues (Zanjani et al. 1998) showed that human BM $CD34^-$ Lin^- cells engrafted *in vivo* and gave rise to progeny that included $CD34^+$ cells. John Dick's group (Bhatia et al. 1998) showed that cells within the $CD34^-$ fraction, with minimum clonogenic/ LTCIC activity, had *in vivo* repopulating activity in NOD / SCID mice. These $CD34^-$ "**SCID repopulating cells**" (SRCs) demonstrated comparable activity to $CD34^+$ SRCs but did not express early HSC markers like Thy-1 or HLA-DR. Goodell and co-workers (Goodell et al. 1996; Goodell et al. 1997) used MoAb to identify a population of cells who despite being $CD34^-$ had marrow-repopulating activity (MRA) in the mouse. These cells were selected on the basis of rapid efflux of the fluorescent Hoechst dye and have been designated side population (SP) cells. Although $CD34^-$ SP cells have been found in humans, MRA for such cells has not been demonstrated (Nakauchi 1998). In summary all the above suggest that there is HSC activity amongst the $CD34^-$ human BM cells however a marker to help identify them is still missing. A recent study (Sato et al. 1999) has shown that, at least in the mouse, CD34 expression reflects the activation state of HPC and that it is reversible (Fig 1.4e). If these findings are confirmed for human stem cells there will be significant implications for human blood stem cell transplantation with respect to CD34 selection in certain clinical situations.

Fig 1.4e CD34 Expression on Murine Haemopoietic Cells. Adapted from (Goodell 1999) and (Huss 1998).

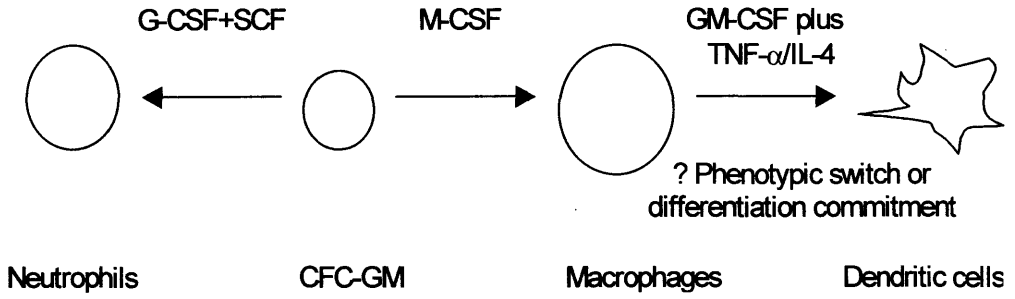


Resting stem cells are CD34 neg/low. After activation they express CD34 and may differentiate or revert back to CD34 neg. CD34neg ($^-$) cells are adherent to the marrow microenvironment whereas $CD34^+$ cells are circulating progenitors.

Lineage commitment and differentiation of HSC

Blood cell production by the BM is controlled by HGFs (Cross et al. 1997), whose role in this is controversial. One school of thought is that stochastic processes primarily mediate HPC differentiation but as the cell becomes lineage restricted it also depends on growth factors (Enver et al. 1998); the other is that HGFs determine lineage commitment (Metcalf 1998a; Metcalf 1998b). Support for the stochastic model comes from i*n vivo* work with HGF/ HGF receptor (HGFR) knockout mice. This has shown that mice lacking GM-CSF were not deficient in myeloid progenitors (Stanley et al. 1994), mice lacking G-CSF had reduced but significant neutrophil production (Lieschke et al. 1994) and Epo/ EpoR knockout mice had near-normal levels of BFU-E (Wu et al. 1995). Secondly expression of cytokine receptors in purified progenitors has been shown to be very low (McKinstry et al. 1997) implying that extrinsic cytokine-mediated signals for such cells are likely to be ineffective (Enver et al. 1998). Lastly expression of a hybrid G-CSF receptor with just an active intracellular domain did not affect lineage commitment (Stoffel et al. 1997). Evidence for extrinsic regulation (reviewed in (Metcalf 1999; Metcalf and Nicola 1995)) comes from the variation in the types and proportions of committed progenitors generated by stem cells with different HGF combinations (Metcalf 1991), the distinct effects of different HGFs on the same progenitor (Metcalf and Burgess 1982) (Fig 1.4f) and the induction of specific differentiation commitment in multipotential cell lines by different CSFs (Heyworth et al. 1990). Commitment in primitive multipotent stem cells may be determined differently from less primitive stem or progenitor cells thus maintaining haemopoietic homeostasis by allowing quick proliferative responses of certain lineages under physiological stresses (bleeding/ infection). So in this setting HGFs may play a more deterministic role in lineage restriction (Enver et al. 1998).

Fig 1.4f Lineage Choice in GM Progenitors depends on Lineage-restricted HGFs



1.5 Haemopoietic stem cell mobilization and PBSC transplantation (PBSCT)

1.5.1 Historical developments

The era of PBSC began in 1909 when Maximow proposed that there were cells in PB capable of maintaining haemopoiesis (Maximow 1909) (Fig 1.5a). However no advances were made in this field until the 1940's when Jacobson showed that rodents could be rescued from lethal irradiation by spleen shielding (Jacobson et al. 1949). Then in 1959 it was proved that the haemopoietic cells allowed for BM recovery (Merwin 1959). The same year dimethylsulphoxide (DMSO) was used successfully as a cryoprotectant (Lovelock and Bishop 1959) and 3 years later Goodman demonstrated the presence of haemopoietic precursors in the PB of animals (Goodman and Hodgson 1962). It was however another 9 years before such cells were shown to be present in humans (McCredie et al. 1971). The numbers of PBSC in normal individuals at steady state conditions were low and their collection for clinical purposes was deemed unpractical. In 1976 however it was discovered that chemotherapy produced dramatic increases in the numbers of GM progenitors in PB (Richman et al. 1976). This together with preclinical studies in dogs (Fliedner et al. 1979; Zander et al. 1984) allowed for the first successful PBSCT in 1985 (Reiffers et al. 1986). Three years later G-CSF was shown to increase PB progenitor numbers (Duhrsen et al. 1988) in cancer patients and in 1989 the first PBSCT was performed using GM-CSF mobilized PBSC (Gianni et al. 1989). By the early 90's G-CSF mobilized PBSC evolved as the preferred source of stem cells for transplantation (Buckner 1999).

Table 1.5a. Major Milestones in the History of PBSC. Adapted and modified from (McCarthy 1993). Important advances in bold.

Year	Event	Reference
1909	**Existence of PBSC proposed**	Maximow
1949-51	Splenic and limb shielding protects rodents from irradiation	Jacobson
	BM infusion protects guinea pigs from irradiation	Lorenz†
1959	DMSO used as cryoprotectant	Lovelock
	PBSC engraft irradiated mice	Merwin
1962	Haemopoietic progenitors in the PB of animals	Goodman
1971	**Haemopoietic progenitors in the PB of humans**	McCredie
1976	**Post-chemotherapy CFC-GM peak identified**	Richman
1979-84	PBSCT studied in dogs	Fleidner, Zander
1980	**Feasibility of progenitor stem cell harvesting**	Korbling†
1983	Improved CFC-GM assay	To†
1985	High-dose alkylating agents	Frei†
1985	**First successful PBSCT**	Reiffers
1988	GM-CSF amplifies post chemotherapy CFC-GM peak	Socinski†
	PBSC mobilized with G-CSF	Duhrsen
1989	PBSCT with GM-CSF mobilized PBSC	Gianni
1992	**PBSCT with G-CSF mobilized PBSC**	Sheridan†

†References not included in the text above, (Frei 1985; Korbling et al. 1980; Lorenz et al. 1951; Sheridan et al. 1992; Socinski et al. 1988; To et al. 1983).

1.5.2 Mobilization of HPC- Animal models

The presence of PBSC in animals was known since 1962 however no attempts were made to pursue this further for clinical use until the mid 1980's because of their low frequency (Georges et al. 1998). Additionally the higher content of T cells in allogeneic animal models discouraged use of PBSC, because of the increased severity of acute GVHD, a major cause of morbidity/ mortality (Storb et al. 1967; Van Bekkum and Lowenberg 1985). Work in canine models however showed that long-term engraftment by allogeneic cells was achievable (Carbonell et al. 1984; Korbling et al. 1979). Abrams and colleagues (Abrams et al. 1981) showed that cyclophosphamide expanded the numbers of HPC in the PB of dogs by ~1 log. Five years later Applebaum used chemotherapy-mobilized autologous PBSC to cure dogs with lymphoma (Appelbaum et al. 1986). A major advancement was the faster haemopoietic reconstitution with PB than BM-derived stem cells (Molineux et al. 1990; Neben et al. 1993; Yan et al. 1995). The first study providing the evidence for the transplantation potential of G-CSF mobilized PBSC in the mouse was by Molineux and co-workers (Molineux et al. 1990). This and other studies on mice showed that HPC could be mobilized into PB and led to the identification of cytokine combinations that mobilized such cells (reviewed in (Georges et al. 1998)).

Overall animal models were important to understand the kinetics of mobilization and the large-animal models allowed us to extrapolate findings to the human (Georges et al. 1998). Such studies predicted the severity of GVHD more accurately and suggested that allogeneic mobilized PBSC achieved higher rates of long-term engraftment than BM; this without the expense of more GVHD, despite the presence of 1 log more T cells in mobilized PB (Georges et al. 1998).

1.5.3 Mobilization of HPC in humans-The role of chemotherapy and cytokines

Fig 1.5a illustrates the changes in PB leucocytes and progenitor numbers following myelosuppresssive chemotherapy. Several cytotoxic drugs alone or in combination produce reversible BM aplasia 7-14 days after administration. Progenitor numbers decline as the leucocyte/ platelet counts drop. CFC numbers begin to rise first and their levels can reach figures up to 100-fold above baseline in normal subjects (To and Juttner 1993). The rebound phase lasts 4-7 days until progenitor numbers normalise (To and Juttner 1993). The precise timing of the leucocyte nadir varies from patient to patient and also for different chemotherapy regimens.

Following Richman's paper in 1976 when the post-chemotherapy CFC-GM peak was identified (Richman et al. 1976) myelosuppressive chemotherapy alone was the first mobilization protocol used. High dose cyclophosphamide was the most frequently used cytotoxic (To et al. 1997). The introduction of r-HGFs in the 1980's led to GM-CSF and G-CSF being the first cytokines to be evaluated. Duhrsen (Duhrsen et al. 1988) and Socinski (Socinski et al. 1988) showed that G-CSF and GM-CSF respectively increased circulating CFU-GM in cancer patients. Since then many studies (Brugger et al. 1992; Elias et al. 1992; Gianni et al. 1990; Haas et al. 1994; Pettengell et al. 1993b; Schwartzberg et al. 1992) examined the role of various cytokine combinations in this setting and suggested that myelosuppressive chemotherapy and HGFs improve mobilization and reduce toxicity. The main HGFs employed include G-CSF, GM-CSF, IL-3, SCF and FLT-3L (summarized in Table 1.5b).

Fig 1.5a CFC-GM and Leukocyte Numbers after Chemotherapy. Adapted and modified from (Brugger et al. 1993).

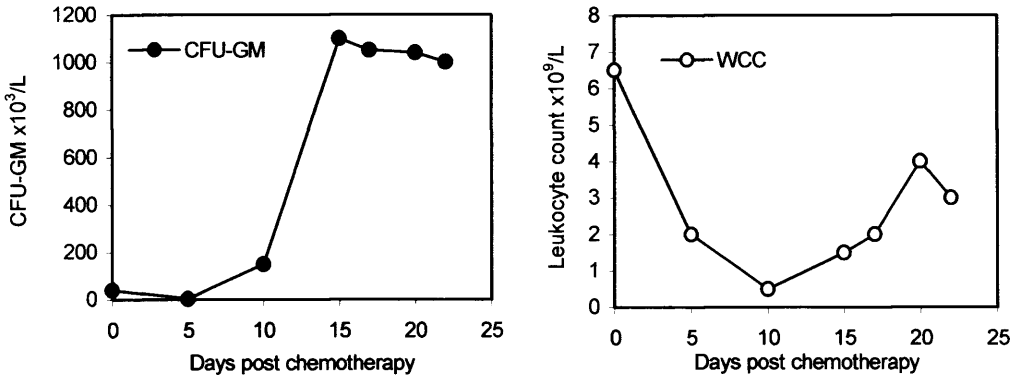


Table 1.5b The Pioneering Studies of Cytokine-Mobilized PBSC in Humans.

Cytokine ± Chemotherapy	Increase in CFC-GM	Study Subjects	Main Outcome of Study	Reference
GM-CSF 4 – 64 μg/ kg/ d (+ Chemo)	13x (63x)	Cancer patients	GM-CSF ± chemo increases CFC-GM	(Socinski et al. 1988)
G-CSF 3 – 5 μg/ kg/ d	100x	Cancer patients	G-CSF increases CFC-GM	(Duhrsen et al. 1988)
GM-CSF plus High-dose Cyclophosphamide	100x	Cancer patients	1st transplant with GM-CSF mobilized PBSC; faster haemopoietic reconstitution†	(Gianni et al. 1989)
G-CSF 12 μg/ kg/ d	58x	Cancer patients	1st transplant with G-CSF mobilized PBSC; faster platelet recovery	(Sheridan et al. 1992)

† Achieved with GM-CSF mobilized PBSC **plus** autologous BM.

G-CSF: This is usually used at 3-6 μg/ kg/ d started the day after chemotherapy is completed until the last day of aphaeresis (Haas et al. 1994; Schwartzberg et al. 1992). For some mobilization protocols a 300 μg ampoule of G-CSF is used for all patients (Haas et al. 1994; Haynes et al. 1995). G-CSF is also used to mobilize PBPC without chemotherapy. This approach is particularly useful for mobilization of PBPC from healthy donors for allogeneic transplantation. The dose used in this setting is usually 10μg/ kg/ d (To et al. 1997). Progenitor numbers increase 40- to 80-fold after 4-5 days (DeLuca et al. 1992; Sheridan et al. 1992) in contrast to 8-12 days needed when G-CSF is used at the lower dose (with chemotherapy) (To et al. 1997).

GM-CSF, GM-CSF and IL-3: GM-CSF is not used routinely now, despite comparable efficacy to G-CSF because of side effects such as fever, hypoxaemia and first-dose reaction (American Society of Clinical Oncology 1994; Lieschke et al. 1990; To et al. 1997). IL-3 used alone has a modest mobilizing effect and it has been tried therefore in combination with GM-CSF or G-CSF. A sequential IL-3/ G-CSF combination was not superior to G-CSF alone (Huhn et al. 1996). IL-3 has significant side effects and is therefore not used routinely for mobilization. PIXY321 is a fusion protein made up of the active domains of GM-CSF and IL-3, which has been used to mobilize progenitor cells (Hohaus et al. 1998).

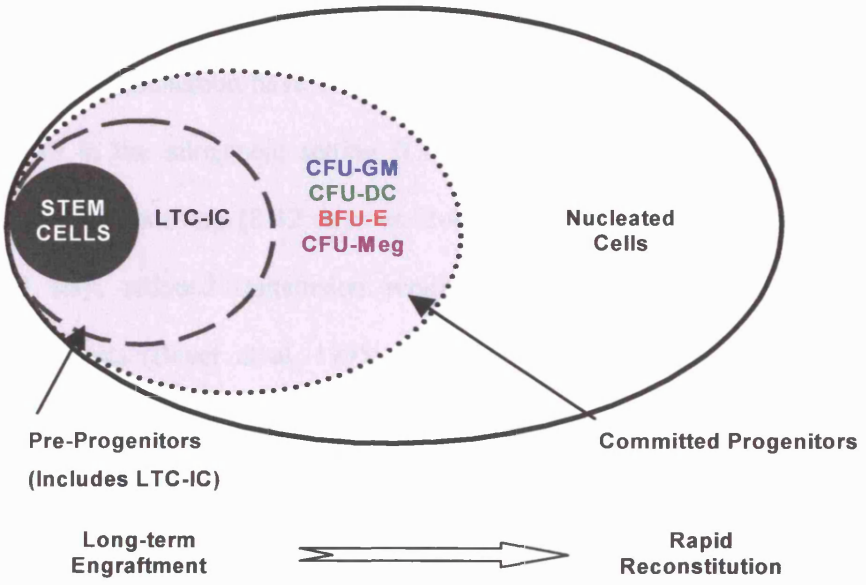
SCF and FLT-3 ligand (FLT-3L): Stem cell factor, the ligand for c-kit, has been shown to have a synergistic effect with G-CSF in the mobilisation of PBPC (Basser et al. 1995; Weaver et al. 1998). Two of the most important effects of SCF when used in combination with G-CSF, were: the dose response relationship for the mobilization of very primitive HPC (LTC-IC) and the reduction in the variability of mobilized LTC-IC with the highest dose levels of SCF (Weaver et al. 1998). A recent randomized trial has shown that the G-CSF/ SCF combination was more effective in mobilizing

HPC than G-CSF alone (Shpall et al. 1999). FLT-3L, the ligand for the tyrosine kinase receptor flk-2/ flt-3 (reviewed in (Lyman and Jacobsen 1998)) has been shown to mobilize PBPC in mice and to have extremely potent synergism with G-CSF (Brasel et al. 1995). Recent work has shown that FLT-3L mobilizes DC progenitors effectively in mice (Maraskovsky et al. 1996) and humans (Maraskovsky et al. 1998).

Other cytokines: Other cytokines that have been tested include human erythropoeitin, IL1, IL-6, IL-8, IL-11 and macrophage inflammatory protein-1 alpha (MIP-1α) (reviewed by (Hohaus et al. 1998; To et al. 1997)).

Timing of aphaeresis and progenitor cell measurement: PBPC mobilization is usually achieved with conventional dose cytotoxic chemotherapy (or high-dose cyclophosphamide) and G-CSF (Olavarria and Kanfer 2000; To et al. 1997); aphaeresis is advised to start when the WCC is 2-5x10^9/ l (To et al. 1997). For allogeneic PBPC collection, from healthy donors, either G-CSF or GM-CSF is used (Cutler and Antin 2001) and aphaeresis is usually done on days 5-7 (d1= first day of G-CSF administration) (To et al. 1997). Guidelines for the collection, storage and progenitor cell measurement of grafts have been developed by the ISHAGE (ISHAGE (International Society for Hematotherapy and Graft Engineeering)- Europe 1997) in association with the European Group for Bone Marrow Transplantation (EBMT). These have been recently updated (Serke and Johnsen 2001). The two parameters, which reflect the haemopoietic reconstitutive capacity of the graft and are most commonly, measured are the CFC-GM content and the number of $CD34^+$ cells. The agreed minimum threshold figures below which haemopoietic reconstitution may not occur are, 15-20x10^4 CFU-GM and 1-2x10^6 CD34 cells per kg body weight (To et al. 1997). Fig 1.5b illustrates the relationships between progenitor cell assays and the engraftment potential of HPC.

Fig 1.5b Progenitor cells, assays and Engraftment Potential. Adapted from (Gee 1998).



Stem cells, pre-progenitors and committed progenitors are $CD34^{+}$. Some stem cells, in the mouse at least, may be CD34 neg/low, as CD34 expression reflects their activation state (Sato et al. 1999) (see section 1.4.4).

1.5.4 Autologous and allogeneic PBPCT and clinical use of cord blood

The increased use of PBPC instead of BM in transplantation is reflected in the data provided by the EBMT group. In 1987 there were 1,000 autologous BM transplants (BMTs) and a few PBPCTs. Nearly a decade later there were > 7,000 PBPCTs and only 800 BMTs (Olavarria and Kanfer 2000). Mobilized PBPC for autologous and allogeneic transplantation have considerable advantages over BM, with the exception of GVHD in the allogeneic setting (Cutler and Antin 2001). Primarily the faster haematological recovery (8-12 days vs. 2-4 weeks after BMT) translates into shorter hospital stay, reduced transfusion requirements, lower morbidity/ mortality and ultimately costs (Beyer et al. 1995; Cutler and Antin 2001; Olavarria and Kanfer 2000; To et al. 1997). Additionally there is avoidance of a general anaesthetic and BM-harvesting associated trauma (To et al. 1997). Finally there is scope for use of PB stem cells for patients where BMT would be too risky or not possible because the BM is fibrotic or involved with tumour (Holyoake et al. 1996).

Over the last 12 years CB has been identified as a potential source of transplantable stem and progenitor cells. Between 1988-1998 ~200 transplant centers from 45 countries have carried out nearly 900 CB transplants (CBTs) (Sirchia and Rebulla 1999). Cord blood has theoretical advantages compared to adult blood cells (Gluckman et al. 1999), whether these are derived from BM or mobilized PB. CB is a rich source of HPC (Broxmeyer et al. 1989; Broxmeyer et al. 1992). CB lymphocytes exhibit a "naive" immunological profile (Roncarolo et al. 1996) with reduced primary T and NK cell cytotoxicity (Gluckman et al. 1999; Sirchia and Rebulla 1999). The naive phenotype of CB lymphocytes and or the lower number of lymphocytes transplanted (Hows 2001) may account for the lower incidence and severity of GVHD observed after CBT than after BMT (Rocha et al. 2000). Understanding more about

the quantitative differences between CB and BM/PB progenitors - especially CFC-DC is important, because one of the many unanswered questions is whether routine engraftment in adults is achievable (~80% CBTs done until 1998 were in recipients < 18 years old (Sirchia and Rebulla 1999)). While many studies have assayed CFC-GM and BFU-E in CB (reviewed in (Broxmeyer 1998; Mayani and Lansdorp 1998)) to date there have been no published data on the number of CFC-DC present per unit volume CB. As discussed in section 1.3, DC are uniquely privileged to control primary and secondary immune responses. Quantifying the committed progenitors of these cells in all the clinically important sources of HPC would be an essential starting point. This would help decide on what is quantitatively the best source of DC progenitors for use in DC-based immunotherapy protocols and how to investigate further the immaturity of the immune system at birth and exploit this with appropriate use of CB HPC for therapeutic use.

1.6 Project objectives

1.6.1 Establishment/ validation of the assay for myeloid DC (chapter 3)

The first aim of the project was the establishment and validation of the clonogenic assay for DC, the CFU-DC (Colony forming unit-Dendritic cell assay). This needed;

- Identification of a batch of serum that supported DC colony growth well.
- Optimization of the growth factor concentrations to maximise DC colony number.
- Optimization of the inoculum, for both $CD34^+$ cells and MNC.
- Evidence of linearity i.e. within a specific range of cells plated there is proportionality between DC colony numbers and numbers of cells plated.
- Confirmation of DC identity using conventional staining and immunocytochemistry.

1.6.2 AC133 and CD33 expression on committed DC progenitors (chapter 4)

Plating $CD34^+$ cells that express the stem cell antigen AC133 in the CFC-DC assay to establish if $AC133^+$ cells preferentially differentiated into DC. This was tested for BM, CB and mobilized PB progenitors. This work was also done using $CD34^+$ cells that expressed the myeloid antigen CD33 to allow for a comparison with the AC133 antigen.

1.6.3 Comparative studies of frequency of progenitors (chapter 4)

A comprehensive comparison of the frequency of CFC-DC in CB, BM and PB was undertaken in order to identify the richest source.

1.6.4 Time course experiments- Optimum time for aphaeresis (chapter 5)

- The assay of CFC-DC and other myeloid progenitors, in steady state and after mobilization.
- The kinetics of mobilization of DC progenitors in patients with solid tumours receiving chemotherapy and G-CSF in order to establish the optimum time window for collection of CFC-DC from mobilized PB.
- The kinetics of mobilization of $AC133^+$ cells in parallel with those of $CD34^+$ cells.
- The DC progenitor content of aphaeresis products.

Chapter 2

General Materials and Methods

Section 1-Cell culture techniques

2.1.1 Media

2.1.1a Iscove's modified Dulbecco's medium (IMDM)

Powder medium (Gibco BRL) was dissolved in 300 ml of double distilled (DD) sterile water and supplemented with 40 ml of 7.5% Sodium Bicarbonate (Sigma), 500U/ml Sodium Benzyl penicillin (Glaxo) and 50μg/ml Streptomycin Sulphate (Sigma). Single strength medium was prepared by adding double distilled sterile water to adjust the final osmolarity to 420-430 mOsm/Kg (~500 ml). Before use for clonogenic assays, cell washings or maintenance of cell suspensions the medium was warmed to 37°C in a water bath and diluted with DD sterile water to adjust the final osmolarity to 320 mOsm/kg (Coutinho et al. 1993). The osmolarity was measured by depression of the freezing point with the Gonotec Osmomat 030 cryoscopic osmometre, Clandon Scientific, calibrated with DD sterile water of 0 mOsm/Kg and a standard saline solution of 300 mOsm/Kg. The medium was filtered and stored at -20°C or at 4°C for up to 2 weeks.

2.1.1b RPMI 1640 medium

RPMI 1640 Medium (Gibco BRL) was used for the culture and maintenance of the 5637-bladder carcinoma cell line. For one litre of medium 100 ml of 10x RPMI 1640 and 29.3 ml of 7.5% Sodium Bicarbonate Solution were added to 870 ml of DD water and supplemented with 500U/ml Sodium Benzyl penicillin and 50μg/ml Streptomycin Sulphate. The pH was adjusted to 7.5 with sterile 1M NaOH (BDH) and then stored at

4°C until required. Immediately prior to use, 1% (v/v) of 200mM L-Glutamine (Sigma) were added.

2.1.1c 10% Stock solution of bovine serum albumin (BSA)

BSA (Sigma) was obtained as powder; stock solutions at 10% (w/v) were prepared as follows: for one litre, 100g of BSA was carefully added to 440ml of double distilled sterile water and left at 4°C for 24 hours until it dissolved completely. The solution was deionised by adding 15g of resin beads (AG 501-X8D, 20-50 mesh, medium porosity, analytical grade; Bio-Rad Laboratories, Richmond, California), stirred slowly at 4°C until all beads have changed colour from yellow to blue. Another 15g of resin beads were added and left stirring overnight at 4°C. The solution was filtered by pouring it through a funnel lined with sterile gauze to catch the beads and then centrifuged at 800g for 30 minutes. An equal volume of double strength IMDM (600mOsm/kg) was added to the final volume of one litre. The BSA solution was sterilised by filtration through a Nalgene 0.45µm membrane filter (Falcon, Becton Dickinson (BD)), aliquoted into 20 ml volumes and stored at -20°C. Prior to use, the BSA solution was split into 5ml aliquots to avoid repeated freezing and thawing. Ten per cent (v/v) of 7.4% Sodium Bicarbonate Solution (Sigma) was added to the 10% BSA solution. It was then added to the growth medium for colony assays to give a final concentration of 1% (Coutinho et al. 1993).

2.1.1d Medium conditioned by 5637 bladder carcinoma cells (5637 CM)

The adherent cells of the bottom of the culture flask were rinsed with RPMI 1640 twice and the washes were discarded; 1.5 ml of 0.05% trypsin plus 5mM EDTA (BDH) were added to cover the adherent layer and the flask placed in a incubator at

37°C for 6-7 minutes, under surveillance. When all cells had detached, the flask was removed from the incubator and 8.5ml of RPMI, supplemented with 20% FCS, were added to make up 10ml of final cell suspension, which was diluted 1 to 10 with RPMI plus 20% FCS and placed into T-75 flasks (Falcon/BD) at 50ml per flask. The flasks were then placed at 37°C for 7 days. The 5637 cells were sub cultured weekly. The conditioned medium was harvested by pouring off the supernatant medium from the culture flasks after one week of incubation and stored frozen at -20°C. It was filtered just prior to use and tested for its ability to support colony growth (Coutinho et al. 1993).

2.1.1e Fetal calf serum (FCS)

Fetal Calf Serum (FCS) was purchased from Sigma (Saint Louis, Missouri) and stored at -20°C. Serum was thawed as needed, filtered, aliquoted and kept at 4°C up to 2-3 weeks. As there is variation in the ability of sera to support colony growth and in the concentration that gives optimal colony numbers (Coutinho et al. 1993), several batches were tested initially until the above batch from Sigma was found to support colony growth well. *This batch of serum (Lot number 26H3396) was therefore used throughout the entire project.* The final concentration of FCS for both the CFC-Mix and CFC-DC assay was 30%.

2.1.1f Methylcellulose

Carboxymethylcellulose powder (Dow Chemical Company, Kent, England) with a viscosity of 4000cps was prepared as a 2.7% stock solution. For one litre, a conical flask with 500 ml of double distilled sterile water heated just below the boiling point was placed on a magnetic stirrer and 27g of methylcellulose powder were dispensed

gently on the surface, stirring continuously. The mixture was then boiled for 10 minutes and left to cool to 37°C before adding 0.5 litre of pre-warmed double strength IMDM. The solution was again placed on a magnetic stirrer and stirred overnight at 4°C to prevent lumps forming. The batch was then frozen to further clarify, as an unclear or lumpy preparation would not allow colony growth. Two aliquots of 15 ml each were placed at 37°C for 7-10 days to check for fungal and bacterial contamination. Before being used for experiments, the batch was thawed, aliquoted in 40ml size Oak Ridge centrifuge tubes (Nalgene), enriched with 10^{-7}M Sodium Selenite (BDH, Poole), 200mM L-Glutamine (Sigma), and $7.5x10^{-5}$M Thioglycerol (Sigma) (all final concentration), allowing sufficient time for these compounds to diffuse through the viscous fluid. The preparation was then centrifuged for 2 hours at 4°C at 17,000rpm (approximately 26,500g) to sediment any particulate matter. Aliquots used for experiments were kept at 4°C and warmed to 37°C before use. Each batch was assayed to determine the optimum viscosity that allowed colony formation. The concentration that supported colony growth varied between 1.2 and 1.3%. *For each set of experiments with a particular sample or patient series the same batch of methylcellulose was used.*

2.1.1g Glutamine, hemin and 2-mercaptoethanol

Stock solutions of these were prepared as follows: A 0.2 M solution of Glutamine was made in DD water/IMDM. This was filtered and stored at -20 °C. Before using, it was warmed to 37 °C in order for it to dissolve completely. A 1M solution of 2-Mercaptoethanol was prepared by diluting a stock 13.5 M solution (Sigma) with DD water. From this 1M solution 50µl were added to 950µl of IMDM (320mOsm/kg) to make a $5x10^{-3}$ M solution. Hemin was available as a stock $2.5x10^{-4}$M solution in

IMDM. Both 2-Mercaptoethanol and Hemin were filtered, aliquoted into 1ml sterile eppendorfs, stored at -20 °C and thawed prior to use.

2.1.1h Cytokines: EPO, GM-CSF, SCF and TNF-α

The cytokines used for clonogenic assays are listed in table 2.1. Stock solutions were made up in PBS/1% BSA and stored at –20°C until use. Following thawing they were kept at 4°C.

Table2.1 Cytokines used for the Multipotent Progenitor† (CFC-Mix) and Dendritic Cell‡ (CFC-DC) Assays.

Recombinant Growth Factor	Concentration	Source
Erythropoietin†	200 U/ ml	Boehringer, Manheim, Germany
GM-CSF‡	1,000 ng/ ml	Glaxo, Geneva, Switzerland
TNF-α‡	1,000 ng/ ml	Sigma, Saint Louis, Missouri, USA
SCF‡	5,000 ng/ ml	Amgen Inc., Thousand Oaks, California, USA

2.1.2 Materials and methods for MNC and HPC isolation

2.1.2a Obtaining blood samples

(i) Bone marrow (BM)

Normal bone marrow samples were obtained either from ribs resected from haematologically normal individuals undergoing thoracotomy or from informed and consenting normal individuals donating their marrows for transplantation. As there was no difference in the culture results of these two sources, data from the two control groups were pooled. The ribs were cut into segments 1-2cm in length by using two 6-inch blunt-ended forceps. Extraneous connective tissue was cut away from the segments which were then immersed in 5-10ml of single strength IMDM supplemented with 2% FCS (v/v). The cells were obtained by repeated flushing using a 5 ml syringe attached to a 21-gauge needle. Single cell suspensions were prepared by passing the suspensions through successively finer gauge needles down to 16-gauge needle to break apart the cell clumps. The cell suspensions were passed through a strainer (Falcon/BD) to separate the small bone pieces were centrifuged at 800g for 10 minutes, the supernatants discarded and the cell pellets resuspended in 5 ml of IMDM single strength supplemented with 2% FCS (v/v) washed twice and kept on ice until required.

(ii) Cord blood (CB)

This was collected aseptically after informed consent into 50ml tubes (Falcon/BD) containing heparin as anticoagulant.

(iii) Peripheral blood (PB)

This was obtained from patients undergoing HPC mobilisation with chemotherapy and rhG-CSF (Lenograstim, Chugai, Japan). For the teratoma patients the dose was 5μg/kg/day and for the breast cancer patients 263μg (equivalent to one 1ml vial) per day in accordance with the high dose therapy protocols for each patient group. Informed consent was obtained from all patients before taking the blood sample (15-20ml) from the site of the intravenous cannula (prior to administration of chemotherapy) or at the time of taking blood for a nadir count. All patients had a full blood count (FBC) 2-3 days before their harvest to check for a rising white cell count (WCC) and again with this a PB sample was taken. For 3-4 samples, which did not necessitate a journey to the hospital, arrangements were made for the blood to be taken locally at the patient's Health Centre. All blood samples were collected in sterile Universal bottles containing 200μl of heparin. Prior to and after collection of the PB the bottles were stored at 4°C. All samples were analysed on the same day.

(iv) Leucaphaeresis products (LP)

These were taken at the end of each collection from the 1-3ml aphaeresis products that remained in the tubing connecting the cell separator centrifuge (Cobe Spectra 3000, Thousand Oaks, California) with the sterile bag where the aphaeresed cells were collected. The LP was collected aseptically in 30ml Universal bottles containing 5ml of single strength IMDM and 200μl of heparin. Samples were processed on the same day. For cell sorting experiments this could not be done (for logistical reasons as the LP samples were obtained in mid-late afternoon) and so the LP were washed three times with sorting buffer, stored overnight at 4°C in 10ml of 40% single strength IMDM, 10% FCS and 50% sorting buffer (v/v) and processed the following morning.

2.1.2b Mononuclear cell (MNC) isolation-Ficoll

All work with human cells was performed in a Class II laminar flow cabinet according to current regulations for safety at work. Blood (BM, CB, PB) was diluted 1:2 with PBS, gently layered onto Lymphoprep 1.077gm/ml (Nycomed, Pharma, Oslo, Norway), and centrifuged at 400g for 25 minutes. MNC obtained from the interface ring were washed once with PBS and resuspended into 1-5ml of cold sorting buffer. This was made and filtered fresh on the day with PBS, 0.005M EDTA and 0.5% (w/v) BSA final concentrations. The cells were than counted and kept on ice until required. LP samples did not need MNC isolation by this method.

2.1.2c Magnetic cell sorting- HPC isolation ($CD34^+$ and $AC133^+$ cells)

$CD34^+$ cells were isolated using the Mini or Midi-MACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly the cell suspension after ficoll was made up to 10ml with sorting buffer centrifuged at 800g for 5 minutes and resuspended in 150µl of sorting buffer per 10^8 cells. Then for every 10^8 cells 50µl of human Sandoglobulin (8mg/ml, Sandoz Pharmaceuticals, Camberley, Surrey) was added followed by 50µl of microbeads conjugated to monoclonal human anti-CD34 (or anti-AC133) antibody, for 30 minutes at 4°C. Thereafter, the cells were washed in cold sorting buffer (5ml/ 10^8 cells), passed through a 40-µm nylon strainer (Falcon, BD) and separated in a Mini -MACS type MS^+ column or a large Midi-MACS LS^+ column (used for $>2x10^8$ cells) exposed to the magnetic field of the Mini-MACS (or Midi-MACS) magnet. The Mini-MACS column was washed four times with 500µl (or three times with 3ml for the larger column) of ice-cold sorting buffer and removed from the separator. Flushing with 1ml of cold sorting buffer using the plunger eluted the cells retained in

the column. For the Midi-MACS column, 5ml of sorting buffer were used to elute the cells. The cells were counted using a haemocytometer, and kept on ice until required.

2.1.2d Cell counting

Cells were counted using an Improved Neubauer Haemocytometer after dilution of 1 to 10 (or higher, if necessary) in white cell dilution fluid. This consisted of 3% glacial acetic acid solution (BDH, Poole) with a few drops of Gentian Violet. The viability of $CD34^+$ or $AC133^+$ cells was always checked by the trypan blue (Sigma) dye exclusion test. An aliquot of the cell suspension and trypan blue (10-50µl) were mixed in 1 to 1 ratio and the number of viable cells (i.e. cells not staining blue) was counted with the haemocytometer. For freshly isolated $CD34^+$ or $AC133^+$ cells this was always >95%.

2.1.3 Assays for colony-forming cells (CFC)

The technique is based on the observation that when haemopoietic cells are immobilised in a semi-solid medium, in the presence of an appropriate source of colony-stimulating factor (either r-HGF or conditioned medium), clonal proliferation and maturation of progenitor cells in that population occurs (Fauser and Messner 1979; Johnson and Metcalf 1977), generating colonies which can be individually scored, thus providing a quantitative measure of the number of different progenitor cells in the cell population under investigation.

2.1.3a Assay for human multipotent progenitor cells (CFC- Mix Assay)

Erythroid (BFU-E, CFU-E) and granulocyte-macrophage (CFC-GM) progenitor cells were assayed in a semi-solid system by a modification of a standard technique as

follows (Coutinho et al. 1993): $5x10^4$ or $1x10^5$ haemopoietic cells from fresh human BM/ CB/ PB/ LP, in single strength IMDM or sorting buffer, were cultured in the assay mixture shown in table 2.2. The inoculum for $CD34^+$ or $AC133^+$ cells was 1-$2x10^3$/ml. The methylcellulose was added to the plating mixture using a 16 gauge blunt needle attached to a 1ml syringe and was mixed thoroughly. The mixture was plated in 3 aliquots of 0.25 ml each, into a 24-wells dish (Falcon, BD) and incubated at 37°C in a fully humidified atmosphere supplemented with 5% CO_2 in air. After 12-14 days, colonies of various sizes and cellular composition could be recognised by direct observation with an Olympus dissecting microscope with a zoom lens at 30x - 40x magnification.

2.1.3b Assay for human myeloid dendritic cell progenitors (CFC-DC)

Myeloid DC progenitors were assayed using a method developed by Young and colleagues (Young et al. 1995) with only minor changes. The basis of the assay, summarized in table 2.3, is the same as for the CFC-Mix assay apart from the culture medium used.

Table 2.2 Composition of the Culture Medium for the CFC-Mix Assay

Final Concentration	Volume added/ assay
30% FCS	300µl
10% deionised BSA	100µl
10% Conditioned Medium	100µl
Cell Suspension	2 to 20µl
2U/ ml Erythropoietin	10µl
1.2% Methylcellulose	500µl
Total volume	**1,012-1,030µl**

Table 2.3 Composition of the Culture Medium for the CFC-DC Assay

Final Concentration	Volume added/ assay
30% FCS	300µl
15% IMDM (320mOsm/ kg)	151µl
$2x10^{-3}$ L-Glutamine	10µl
$5x10^{-5}$ 2-Mercaptoethanol	10µl
$2.5x10^{-6}$ Hemin	10µl
Cell Suspension	2 to 20µl
10ng/ ml GM-CSF	10µl
20ng/ ml SCF	4µl
5ng/ ml TNF-α	5µl
1.3% Methylcellulose	500µl
Total volume	**1,002-1,020µl**

2.1.3c Scoring colonies derived from progenitor cells

Clones of 50 or more cells were scored as colonies (Metcalf 1977). The colonies were defined as follows: CFC-GM, colonies containing granulocytes and/or macrophages; BFU-E, colonies containing three or more clusters of erythroid cells; CFU-E, colonies with 1-2 clusters of erythroid cells; CFC-Mix, colonies containing more than one lineage of cells including erythroid.

2.13d Scoring dendritic cell colonies

Clones of 50 or more cells were scored as colonies. The colonies were defined as follows: Dendritic cell colonies were of two types, either pure DC colonies or mixed DC-Macrophage/Granulocyte colonies (if these cell types were present in approximately equal proportions). These two colony types were scored separately although in the data analysis their numbers were added to give the total CFC-DC figure. Granulocytes/macrophage colonies identical to those seen in the CFC-Mix assay were also scored.

2.1.4 Validation of the CFC-DC assay

It was important to find out the optimum number of cells for which a linear relationship existed with the number of colonies obtained. For the CFC-Mix assay the optimum inoculum is 5-10x10^4 MNC and 1-2.5x10^3 $CD34^+$ cells. This needed to be tested for the DC assay and so linearity experiments were carried out to establish if there was a linear relationship between the number of cells plated and the number of DC colonies obtained.

2.1.4a Linearity experiments

In preliminary experiments MNC were plated at 1-8 x 10^5 cells per ml however this was found to make scoring difficult because of excessive colony growth; moreover DC colonies did not grow well and when they did, they could not be reliably identified if more than $1x10^5$ MNC were plated. This was also seen when $CD34^+$ cells were plated at $2x10^3$ cells and above. Therefore the inoculum for both MNC and $CD34^+$ (or $AC133^+$) cells was reduced and the linearity experiments were repeated as follows:

- MNC plated at $1x10^4$, $2.5x10^4$, $5x10^4$ and $10x10^4$ per ml per assay.
- $CD34^+$ (or $AC133^+$ cells plated at $0.5x10^3$, $1x10^3$, $2x10^3$, and $4x10^3$ and $5x10^3$ per ml per assay.

2.1.4b Dose response experiments

To establish the optimum cytokine concentrations for the generation of DC colonies I tested the response of $CD34^+$ cells over a broad range of different TNF-α and GM-CSF concentrations. TNF-α was tested initially at 2,5,10 and 20ng/ml and later on at 0, 0.5, 1, 2, 5, 10 and 20ng/ml. GM-CSF was tested at 0, 5, 10, 50, 100 and 200 ng/ml. In addition for each batch of TNF-α dose response experiments were done.

Section 2-Cell staining techniques

2.2.1 Media

2.2.1a Fixatives: Paraformaldehyde, acetone/ methanol

Paraformaldehyde was used as a 4% solution (w/v) made up with PBS. 4gm of paraformaldehyde powder (Aldrich, Poole) was added to 100ml of sterile PBS, which was heated gently (inside a fume cabinet) to 100°C to help it dissolve. After cooling down the paraformaldehyde was stored at 4°C for up to one month. Acetone and Methanol (both reagents from Prolabo, Fontaney, France) were made fresh on the day of use and mixed in a ratio of 1:1.

2.2.2 Conventional staining

2.2.2a Pooling of colonies and cytospin preparation

To examine the morphology of colony cells, isolated colonies generated in semi-solid cultures were plucked with a finely drawn-out Pasteur pipette under an inverted microscope and transferred to a solution of single strength IMDM and 10% BSA in 1:1 ratio. After pooling 50-100 colonies the cell suspension was centrifuged at 400g for 5 minutes and resuspended in 2ml of fresh IMDM/ BSA solution. The cell suspension was added into a plastic carrier and centrifuged against APES coated slides with the edges covered with absorbent paper at 500 rpm for 5 minutes in a Shandon-Cytospin 2 centrifuge. The slides were air dried and stained as required.

2.2.2b May-Grunwald Giemsa staining

This technique was applied to fresh and cultured cells. May-Grunwald (Gurr/ BHD) stain was layered onto slides for 3 minutes and rinsed off with tap water. The slides were then coated with Giemsa stain (Gurr/ BHD) diluted 1:20 in DD water for 15 minutes. After washing in tap water, the slides were air-dried mounted with De-Pex mounting medium (BDH, Poole) and the films examined in an Olympus BH-2 microscope using oil immersion lenses (600-1000x).

2.2.3 Immunohistochemistry

2.2.3a The avidin biotin peroxidase complex (ABC) method

Cytospin slides for immunohistochemistry were marked with a wax pen (Dako) around the cytospin. The cells were fixed either 4% paraformaldehyde for 15 minutes or a mixture of acetone/methanol (1:1 ratio) for 90 seconds. The type of fixative depended on the primary antibody used (please see chapter 3). Once fixed the cells were washed with PBS three times. The staining from this stage onwards was done in a humidified atmosphere by placing the slides on a rack inside a flat plastic container containing tissue paper soaked with sterile water. The primary antibody (table 2.4) was diluted to a final protein concentration of ~2μg/ ml with Dako diluent (Dako, Glostrup, Denmark) and 1% human group AB serum. An isotype-matched control (table 2.4) was also used at the same protein concentration as the primary antibody. Both were applied on the cells for one hour. Following three washes with PBS a solution of 9.8ml PBS with 0.1ml azide (1.5M) and 0.1 ml of 30% Hydrogen peroxide (BDH, Poole) was put on the cells for 10-15 minutes. After three more washes with PBS a solution of "RAMBO", rabbit anti-mouse biotinylated antibody (Dako), was applied (1/400 dilution, made with Dako diluent and 1% human AB serum). Following a 40-minute incubation period and three more washes with PBS the ABC complex was put on the cells (9μl of avidin and 9μl of biotin for every 1ml of double distilled water) for a further 40 minutes. A solution of DAB (3,3′-diaminobenzidine, Sigma) was made by adding one gold and one silver tablet to 5ml of DD water and this was put on the cells for 5-15 minutes. This was washed with DD water and the cells were then counterstained as described below.

Table 2.4 Monoclonal Antibodies used for Immunohistochemistry

CD number	Concentration used[†]	Clone	Isotype	Source
CD83	1:100	HB15a	IgG2b kappa (Mouse)	Immunotech, Marseille, France
CD86	1:500	BU63	IgG1 (Mouse)	Serotec, Oxford, UK
HLA-DP-DR	1:100	CR3/43	IgG1 kappa (Mouse)	Dako, Glostrup, Denmark

[†] Primary antibody diluted to achieve a final concentration of ~2μg/ ml

Table 2.5 Isotype-Matched Control Antibodies[†] used for Immunohistochemistry

Control Antibody	Clone	Isotype	Source
IgG2b Negative Control (Used for CD83)	DAK-GO9	IgG2b kappa (Mouse)	Dako, Glostrup, Denmark
IgG1 Negative Control (Used for CD86 and HLA-DP-DR)	DAK-GO1	IgG1 kappa (Mouse)	Dako, Glostrup, Denmark

[†] Control Antibodies diluted to achieve a final concentration of ~2μg/ ml

2.2.3b Counterstaining

This was done in a class 1-safety cabinet. The slides were counterstained with Gills 2x haematoxylin for 3 minutes, washed in running water for 1 minute followed by 3 dips in alkaline water and one more wash in running water for 1 minute. The cells were then dehydrated by six dips through each of the following: 70% alcohol, 90% alcohol, 100% alcohol, 100% alcohol, 100% alcohol, and xylene (2 troughs). Finally the cells were mounted and allowed to dry overnight before examined in an Olympus BH-2 microscope using oil immersion lenses (600-1000x).

Section 3- Flow cytometry

2.3.1 Media

2.3.1a Lysing solution

This was prepared by adding one vial of Orthommune (Ortho Diagnostic Systems, Raritan, New Jersey) to 100 ml of millipore water. The solution was stored at room temperature for up to a month.

2.3.1b Monoclonal antibody conjugates used

These are listed in table 2.6 and the isotype-matched controls in table 2.7.

Table 2.6 List of Monoclonal Antibody Conjugates used

CD number	Common Name	Clone	Isotype	Conjugate	Source
CD45	Anti-HLe-1	2D1	IgG1	FITC[†]	BD[§]
CD34	Anti-HPCA2	8G12	IgG1	PE[‡]	BD
CD34	Anti-HPCA2	8G12	IgG1	FITC	BD
AC133	-	-	IgG1	PE	AmCell Inc.
AC133-1	AC133/I-PE	AC133-1	IgG1	PE	Miltenyi
CD33	Leu-M9	P67.6	IgG1	PE	BD

[†]FITC, Fluorescein isothiocyanate; [‡]PE, Phycoerythrin; [§]BD, Becton Dickinson

Table 2.7 List of Mouse Immunoglobulin Fluorescence Controls used

Control Antibody	Clone	Isotype	Conjugate	Source
γ_1	X40	IgG_1	FITC	BD
γ_1	X40	IgG_1	PE	BD

2.3.2 $CD34^+$ and $AC133^+$ cell analysis in whole blood samples

CD34 antigen expression was studied using phycoerytrhin (PE)-conjugated CD34 monoclonal antibody (MoAb; anti-HPCA2, Becton Dickinson, Cowley, Oxon, UK) and AC133 expression using a PE-conjugated AC133-1 MoAb (Miltenyi Biotec, Bergisch Gladbach, Germany). To 50 μL of whole blood samples (or LP volume containing 1 x 10^6 MNC), 10 μL of directly conjugated CD34 or AC133 MoAb was added and mixed with 10 μL of fluorescein isothiocyanate (FITC)-conjugated CD45 MoAb (anti-HLe-1, Becton Dickinson) for 15 minutes at room temperature. An isotype matched irrelevant antibody was used as a control. Then 2ml of lysing solution was added and after 10 minutes incubation the cells were centrifuged, resuspended with PBS washed and fixed in 1% formaldehyde. The samples were analysed on a FACS Vantage flow cytometer equipped with a 488nm Argon laser (Becton Dickinson). $CD34^+$ and $AC133^+$ cells were enumerated using the ISHAGE guidelines (Sutherland et al. 1996). The PC LYSYS software programme (version 1.1) was used for all analyses. Briefly cells were gated into region 1 (R1), which included all, nucleated leucocytes but excluded red cells, nucleated red cells, platelets and cellular debris. Then $CD45^+$ events within R1 were analysed for CD34 staining and positive $CD34^+$ events cells were gated into region 2 (R2). Events defined by R1 and R2 ($CD45^+$ and $CD34^+$) were displayed on a dot plot with the light-scatter (side scatter) features of cells on the y-axis and CD45 fluorescence on the x-axis. Cells with low side scatter and low-intermediate CD45 fluorescence were gated within region R3. This excluded events like debris, platelet aggregates and mature myeloid cells. Finally the forward scatter was plotted against the side scatter properties of cells from regions R1+R2+R3. Cells with uniformly low side scatter were gated in region R4 and included in the percentage of $CD34^+$ cells. The same gating strategy was applied

for the CD34-isotype CD45 control. The same method was also applied to cells stained with PE-conjugated AC133 MoAb. For PB and LP samples an automated differential leukocyte count was checked to allow for precise enumeration of $CD34^+$ and $AC133^+$ cells. The percentage difference between $CD34^+$ cells minus $CD34^+$ events in the control was multiplied by the total leucocyte count to determine the number of $CD34^+$ cells per ml PB.

2.3.3 CD34 analysis of immunomagnetically separated cells

To check the purity of $CD34^+$ separated cells, two aliquots of $2\text{-}5x10^5$ cells each were centrifuged at 800g for 5 minutes, resuspended in 50µl sorting buffer; 10µl of CD34 PE-conjugated MoAbs and 10µl of an isotype matched control (IgG_1) were added to each of the samples and incubated in the dark, at room temperature for 10-15 minutes. The labelled aliquots were washed once in sorting buffer, the pellets were resuspended in 300µl of 1% formaldehyde and analysed on the flow cytometer. The samples were analysed on the same FACS Vantage flow cytometer as the one used for $CD34^+$ and $AC133^+$ cell analysis in whole blood samples (PC LYSYS software version 1.1). Cells labelled with the CD34 PE-conjugated MoAb were displayed using dot plots where the light-scatter (side scatter, SSC) features of cells were plotted against the forward scatter (FSC) features –see appendix A. Events were then gated into region R1, which included all, nucleated leucocytes but excluded red cells, nucleated red cells, platelets and cellular debris. Events defined by R1 were displayed on a second dot plot with the light-scatter (side scatter) features of cells on the x-axis and CD34 fluorescence on the y-axis. The same process was applied to cells labelled with the isotype control. The purity of $CD34^+$ cells (expressed as a %) was determined by the difference between, the percentage of cells showing CD34 fluorescence ($>10^1$

log scale) and uniformly low side scatter (SSC<200) –defined by events in the upper left quadrant of the second dot plot- in the CD34-PE labelled and isotype control-PE labelled samples.

2.3.4 Cell sorting

Immunomagnetically isolated $CD34^+$ cells were split into two aliquots. One was labelled with FITC-conjugated anti-human CD34 and AC133 (or CD33) and the other one with isotype matched irrelevant MoAbs in a total volume of 100µl. Cells were incubated for 15 minutes at room temperature washed with PBS and resuspended in 900 µl and 250 µl for the positively labelled aliquot and the control respectively. The cells were sorted into AC133 (or CD33) positive and negative fractions under sterile conditions using the Automatic Cell Deposition Unit (ACDU) on a FACS Vantage flow cytometer (Becton Dickinson). The sorted cells were suspended in an ice-cold solution of IMDM with 10% FCS and kept on ice until plating. A total of 1-2 x 10^3 cells of each fraction were plated in colony forming assays (CFC-DC and CFC-Mix as described earlier). For the method used to determine the relative percentages of double positive and single positive cells within the sorted CD34 population please see appendix B.

Section 4- Statistical Analysis

2.4.1 Statistics

The computer programme SPSS student version 10.0 was used for statistical analysis. For the analysis of AC133 expression on $CD34^+$ cells the significance of the medians was assessed with the Kruskal-Wallis test. The Mann-Whitney test was used to verify differences in progenitor cell content between $AC133^+$ and $AC133^-$ cells, and

$CD33^+$ and $CD33^-$ cells and to compare the incidence of CFC-DC, CFC-GM and BFU-E, between the different sources of HPC used, i.e. BM versus CB, BM versus mobilized PB, and CB versus mobilized PB. The numbers of progenitor cells per ml of CB and mobilized PB were compared using the Mann-Whitney test. Progenitor numbers in PB, at steady state and after mobilization were compared using the Wilcoxon matched-pairs signed-rank sum test. The Kruskal-Wallis test was applied to compare progenitor numbers in PB, before the first cycle of chemotherapy to those before cycle 2 and after mobilization.

Chapter 3

Establishment and Validation of the CFC-DC Assay

3.1 Introduction

The first clonogenic assay, which allowed enumeration of DC progenitors in BM and mobilized PB, used non-adherent MNC (Reid et al. 1990). The assay developed by Young and colleagues (Young et al. 1995) employed $CD34^+$ cells. The study of the mobilization kinetics of DC progenitors (please see chapter 5) required blood sampling at both steady state and after mobilization. The only way to assay progenitors in both these states was to plate MNC at all time points of the study. This was because the number of $CD34^+$ cells that could be obtained, using immunomagnetic separation, from a relatively small volume (10-25 ml) of PB at steady state or in the early days of mobilization was too low. Even though the number of $CD34^+$ cells needed for plating in the CFC-DC and CFC-Mix assays was small (2-3 $x10^3$), a minimum of $1x10^5$ cells was required for checking the purity of the separated cells using flow cytometry. This was particularly important when starting with relatively low numbers of MNC and/ or when the percentage of $CD34^+$ cells in whole blood was low (<0.1%). Adequate numbers of $CD34^+$ cells could be obtained only when their percentage in PB had increased from 0.01-0.09 (steady state) to at least 0.1% and preferably >0.2%.

In order to demonstrate that the number of colonies was proportional to the number of cells plated linearity experiments were conducted with MNC, $CD34^+$ cells and $AC133^+$ cells from BM, mobilized PB and CB. The objective was to show that within a specified range of cells plated, the number of DC colonies was proportional to the inoculum. With regards to optimum culture conditions the two most important

cytokines required for myeloid DC development/maturation *in vitro* are TNF-α and GM-CSF (Caux and Banchereau 1993; Caux et al. 1992). Therefore dose response studies were undertaken to establish the optimum concentrations of these vital cytokines for the growth of $CD34^+$ DC progenitors from BM, mobilized PB and CB. Finally morphological confirmation of the identity of the cells grown in the DC assay was undertaken. This involved inspection of the colonies using inverse light microscopy and May-Grünwald Giemsa staining of DC isolated from day 12-14 colonies. In addition immunocytochemistry of these cells was carried out using three of the most important and commonly used markers that identify mature DC; namely CD83, CD86 and MHC-class II molecules (HLA-DP-DR) (Zhou and Tedder 1995a).

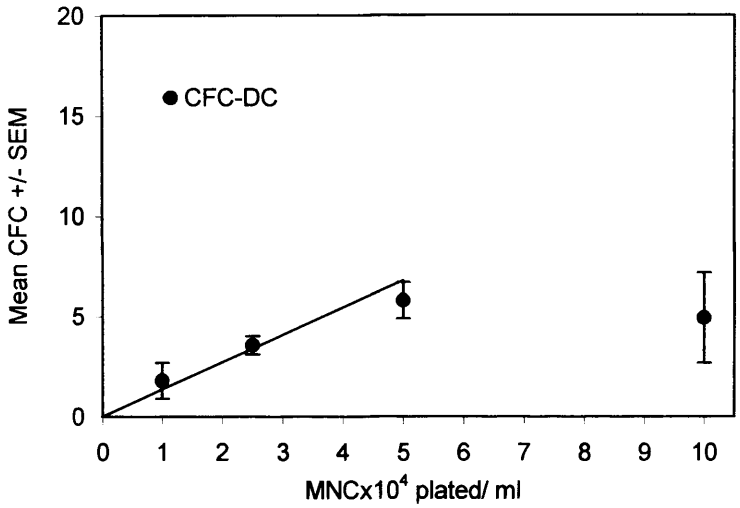
3.2 Results

3.2.1 Linearity experiments

(i) Linearity with MNC from BM, CB and PB

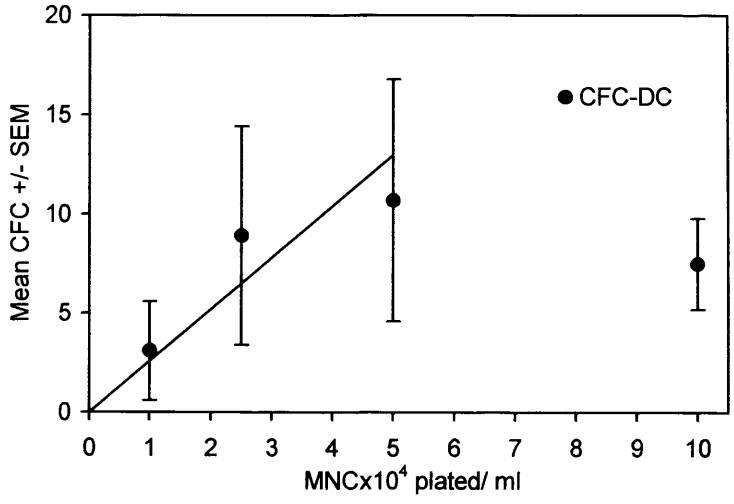
In BM the number of CFC-DC was proportional to the number of MNC plated when the inoculum was $1\text{-}5x10^4$ per ml (Fig 3.1). Similar findings were seen with MNC from CB (Fig 3.2) and mobilized PB (Fig 3.3). All three graphs show that when the inoculum was $5x10^4$ MNC or less there was a linear relationship with the numbers of DC colonies obtained. For CFC-GM however linearity was maintained with up to $1x10^5$ MNC (Fig 3.4).

Fig 3.1 Linearity in CFC-DC with BM Mononuclear Cells.



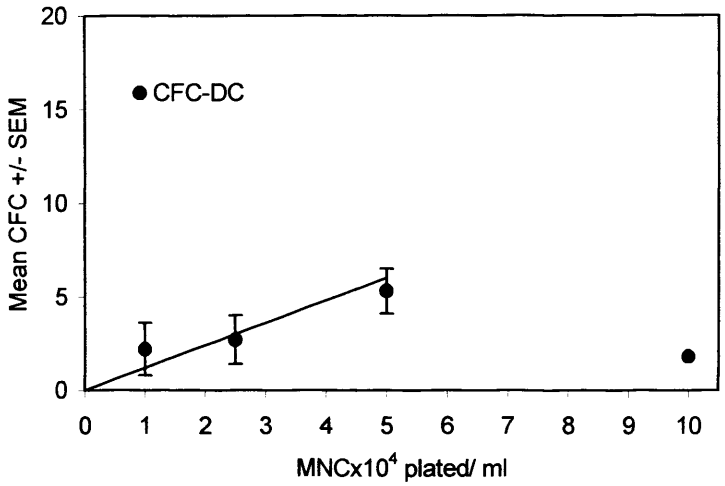
Linearity observed with up to $5x10^4$ MNC/ ml. The data show no linearity beyond $5x10^4$ cells. The data are the mean of three separate experiments ±SEM.

Fig 3.2 Linearity in CFC-DC with CB Mononuclear Cells.



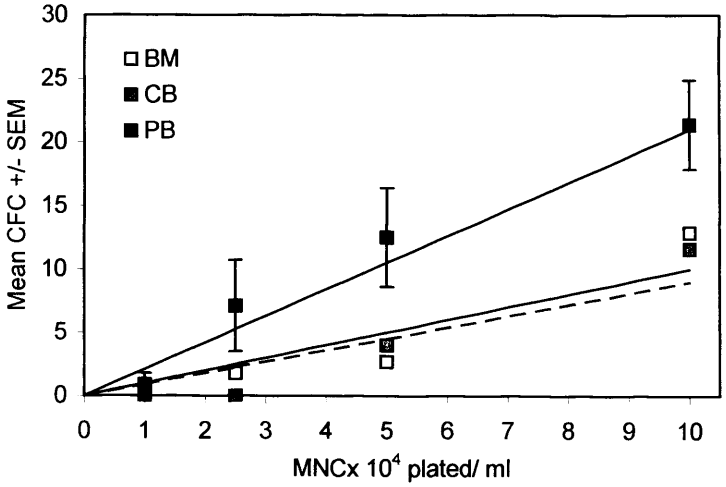
Linearity observed with up to $5x10^4$ MNC/ ml. There was no linearity beyond $5x10^4$ cells. The data are the mean of three separate experiments ±SEM.

Fig 3.3 Linearity in CFC-DC with Mobilized PB Mononuclear Cells.



The straight line shows there was no linearity beyond $5x10^4$ cells/ ml. The data are the mean of three separate experiments ±SEM.

Fig 3.4 Linearity in CFC-GM with BM, CB and Mobilized PB Mononuclear Cells.



The graph shows linearity for GM colonies in the DC assay beyond $5x10^4$ cells. The bottom solid line is for the BM samples and the broken line for the CB samples. Only the error bars for the PB samples have been included for purposes of clarity. The data are the mean of three separate experiments ±SEM.

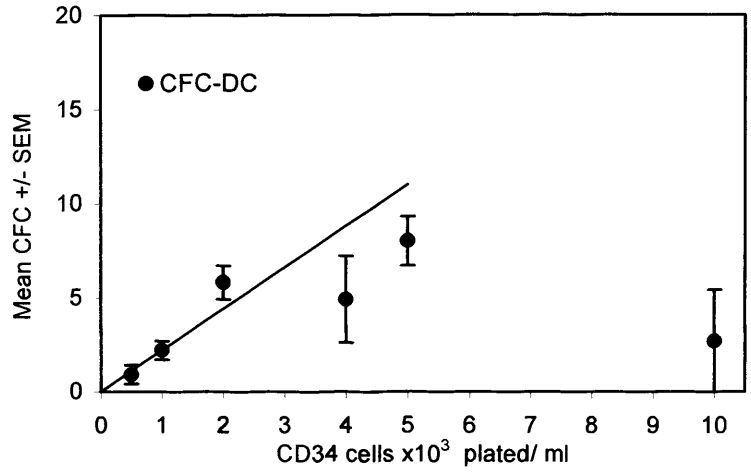
(ii) Linearity with $CD34^+$ cells from BM, CB and PB

For $CD34^+$ cells linearity in the number of CFC-DC generated was observed between 0.5-2x10^3 cells. This was seen for BM (Fig 3.5), CB (Fig 3.6) and mobilized PB (Fig 3.7). When 4-5x10^3 cells were plated the number of CFC-DC did not increase as expected but instead declined. For BM when a maximum of 1x10^4 $CD34^+$ cells was plated the number of DC colonies obtained was even lower than when 5x10^3 cells were plated. CB $CD34^+$ cells generated, roughly, double the number of DC colonies compared to BM when plating 0.5-2x10^3 cells while mobilized PB gave slightly higher numbers of DC colonies than CB when plating 0.5-2x10^3 cells. The percentage purity of immunomagnetically separated $CD34^+$ cells has been rounded off to the nearest decimal point.

(iii) Linearity with $AC133^+$ cells from BM, CB and PB

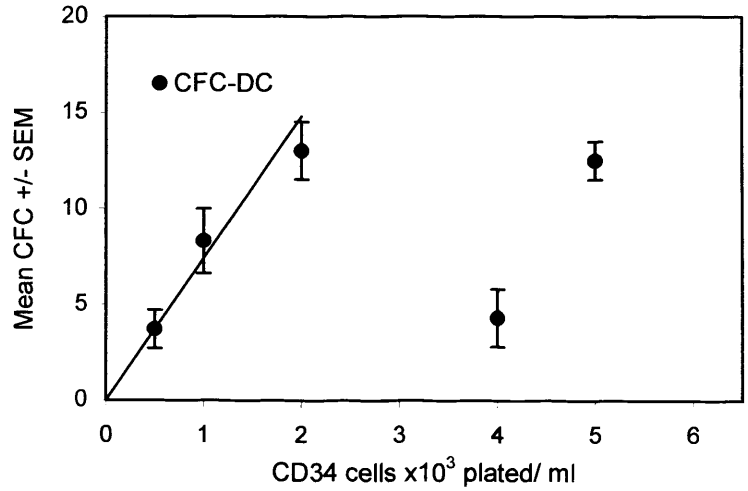
For $AC133^+$ cells linearity in the number of CFC-DC generated was observed between 0.5-2 x10^3 cells in BM (Fig 3.9) or CB (Fig 3.10) and 0.5-1 x10^3 cells for mobilized PB (Fig 3.11). When 4-5 x10^3 cells were plated, again the number of CFC-DC did not increase as expected but instead declined. For BM when a maximum of 10x10^3 $AC133^+$ cells was plated, DC colony growth was markedly suppressed. The purity of the AC133 separated cells could not be checked when these experiments were done. This was because only one MoAb against the AC133 antigen was available at the time. AC133 expression of the "minimacs" separated cells as assessed by the PE-conjugated MoAb (AmCell Inc., USA) was low, presumably because "both Monoclonals" (i.e. the one conjugated to the microbeads and the fluorochrome conjugate) recognised the same epitope (Miltenyi-Biotec 1998).

Fig 3.5 Linearity in CFC-DC with BM $CD34^+$ Cells.



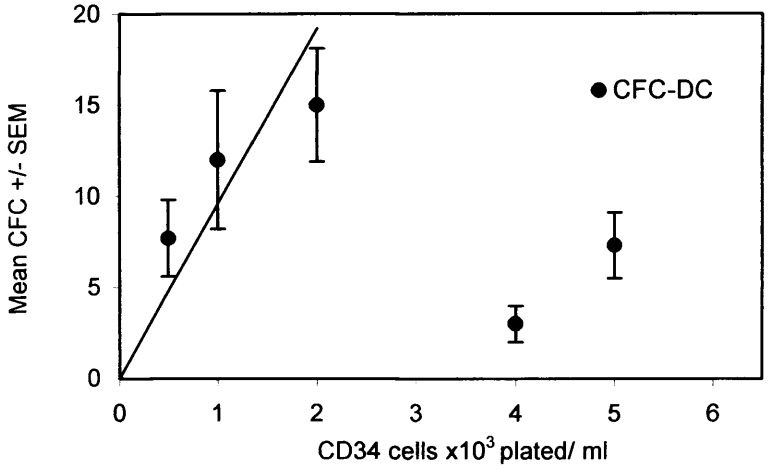
There was no linearity beyond 2,000 cells/ ml. The data are the mean of three separate experiments ±SEM. The mean purity of the $CD34^+$ cells was 86% (SEM ±3%).

Fig 3.6 Linearity in CFC-DC with CB $CD34^+$ Cells.



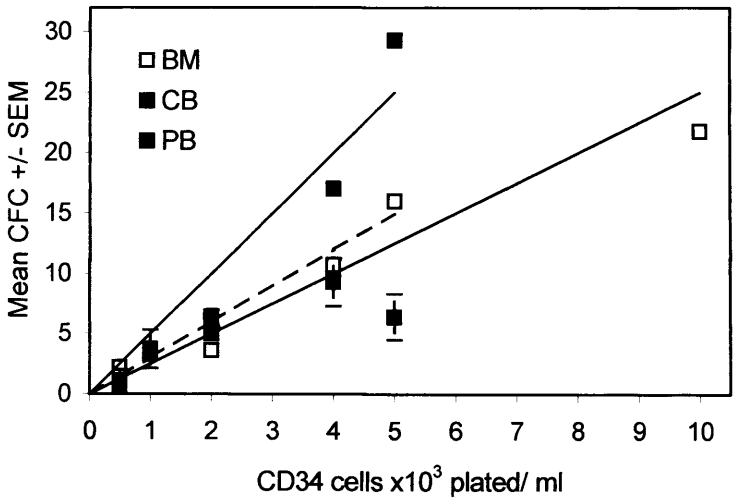
There was no linearity beyond 2,000 cells/ ml. The data are the mean of four separate experiments ±SEM (n=4, for $5x10^3$ cells n=3). The mean purity of the $CD34^+$ cells was 73% (SEM ±11%).

Fig 3.7 Linearity in CFC-DC with Mobilized PB CD34$^+$ Cells.



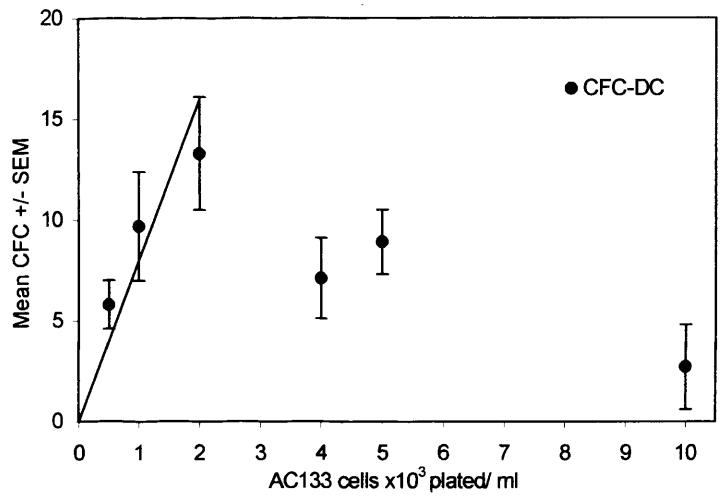
There was no linearity beyond 2,000 cells/ ml. Mean of four separate experiments ±SEM (n=4; for 5x10^3 cells n=3). Mean purity of the CD34$^+$ cells, 82% (SEM ±4%).

Fig 3.8 Linearity in CFC-GM with BM, CB and Mobilized PB CD34$^+$ Cells.



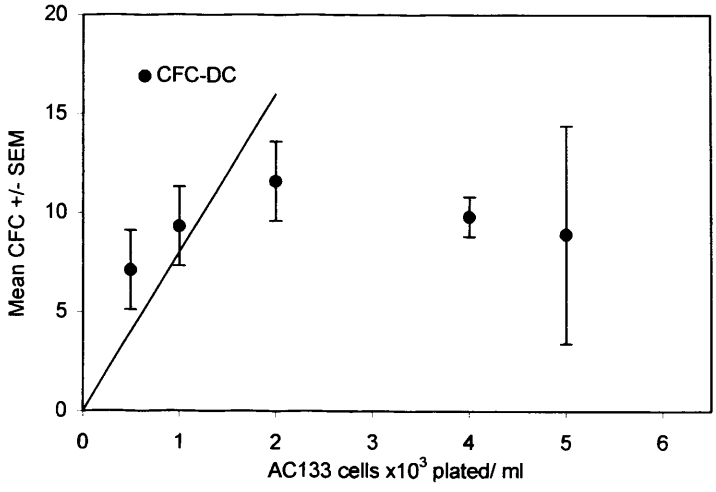
Linearity for GM colonies in the DC assay with up to 5x10^3 cells/ ml. The top *(solid)* line is for CB (n=4; for 5x10^3 cells, n=3). The bottom *(solid)* line and the middle (broken) lines are for BM (n=3) samples and PB (n=4; for 5x10^3 cells, n=3) respectively. Only the error bars for the PB samples have been included for purposes of clarity.

Fig 3.9 Linearity in CFC-DC with BM AC133$^+$ Cells.



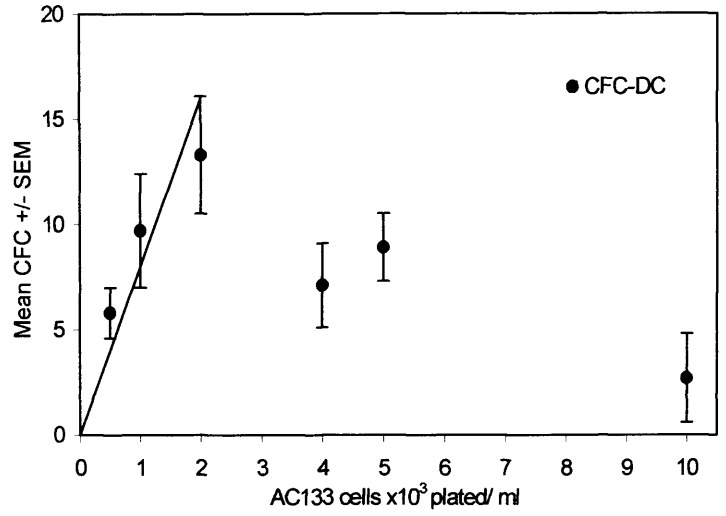
The straight line shows loss of linearity beyond 2,000 cells/ ml. The data are the mean of three separate experiments ±SEM.

Fig 3.10 Linearity in CFC-DC with CB AC133$^+$ Cells.



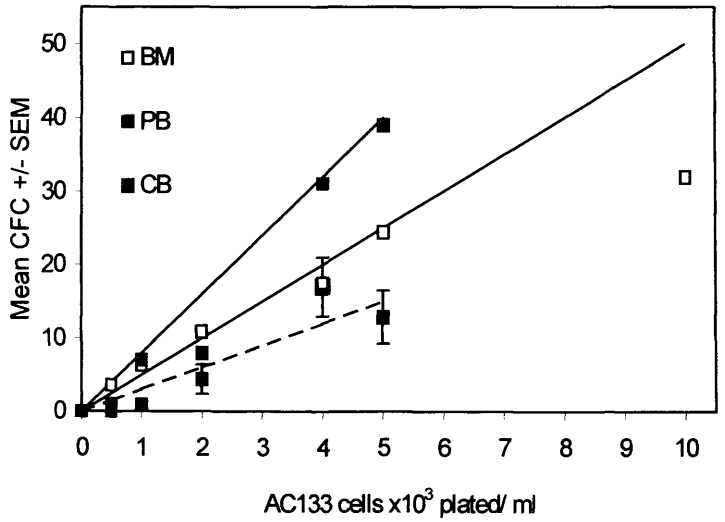
There was no linearity beyond 2,000 cells/ ml. The data are the mean ±SEM from three separate experiments.

Fig 3.11 Linearity in CFC-DC with Mobilized PB AC133$^+$ Cells.



The straight line shows loss of linearity in this case beyond 2,000 cells/ ml. The data are the mean of three separate experiments ±SEM.

Fig 3.12 Linearity in CFC-GM with BM, CB and Mobilized PB AC133$^+$ Cells.



The graph shows linearity for GM colonies in the DC assay up to 5x10^3 cells/ ml. The top and middle lines are for CB and BM samples respectively and the broken line is for PB. The data are the mean of three separate experiments ±SEM.

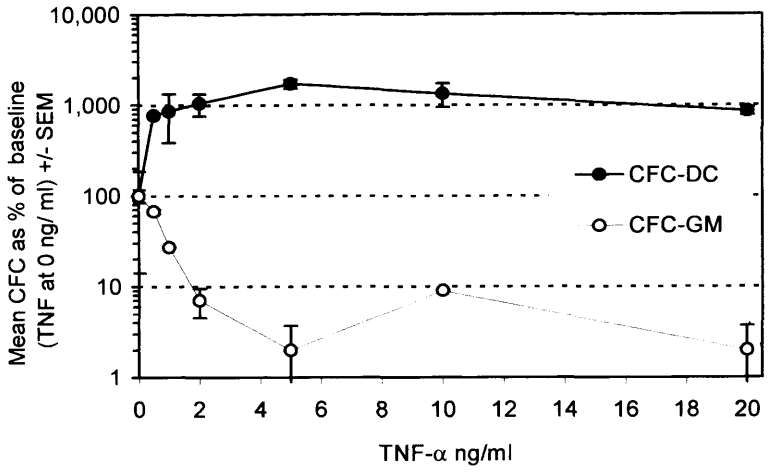
3.2.2 Dose response experiments: (i) TNF-α

Dose response of CD34$^+$ cells from BM to TNF-α: In preliminary experiments where $5x10^3$ $CD34^+$ cells were plated it was shown that the optimum concentration of TNF-α was 5ng/ ml (data not shown). These experiments were repeated once it became clear that the optimum number of cells to be plated was $1\text{-}2x10^3$. Initially TNF-α was used at four concentrations (2, 5, 10 and 20 ng/ml). These showed clearly that 5 ng/ ml gave the highest number of DC colonies (n=3, data not shown). To ensure that concentrations of TNF-α below 2ng/ ml did not generate higher numbers of DC colonies the dose response experiments were repeated using TNF-α at 0, 0.5, 1, 2, 5, 10 and 20 ng/ ml (Fig 3.13). These doses inhibited growth of CFC-GM.

Dose response of CD34$^+$ cells from mobilized PB to TNF-α: The response of PB $CD34^+$ cells to TNF-α was similar to that of BM $CD34^+$ cells. Dose response experiments (range 0, 0.5, 1, 2, 5, 10 and 20ng/ ml) confirmed the optimum TNF-α concentration to be 5ng/ ml (Fig 3.14).

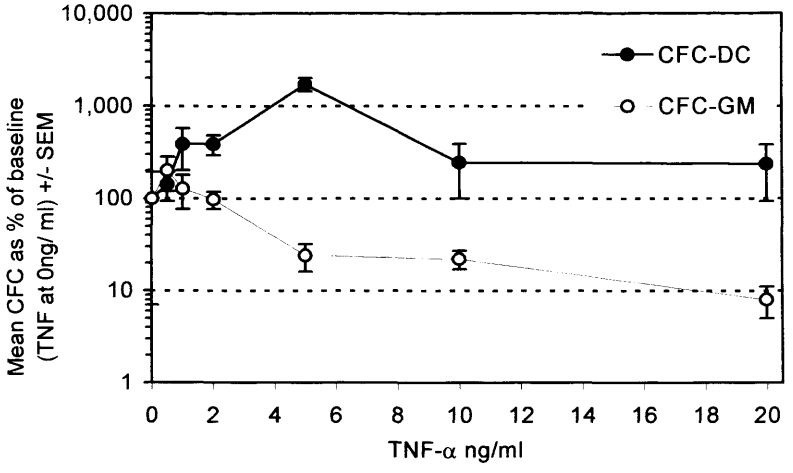
Dose response of CD34$^+$ cells from CB to TNF-α: In preliminary experiments $2x10^3$ $CD34^+$ cells were plated and showed the optimum concentration of TNF-α to be 2ng/ ml (n=4, data not shown). These were repeated using TNF over a wider range 0, 0.5, 1, 2, 5, 10 and 20ng/ ml (n=3, Fig 3.15a). These experiments demonstrated that the optimum concentration was around 1-2ng/ml and beyond 2ng/ml there was a decline of ~35% in CFC-DC numbers. However at the 1-2ng level only about two thirds of the total DC colonies were of the pure type whereas at 5ng/ ml >90% of all CFC-DC were of the pure type (Fig 3.15b). Moreover 5ng/ ml was the concentration used for CD34 cells from BM and PB and would therefore make a comparison with CB CD34 cells more meaningful. Therefore the author plated CB $CD34^+$ cells in the CDC-DC assay using TNF-α at 5ng/ ml.

Fig 3.13 Dose Response of BM CD34$^+$ Cells to TNF-α.



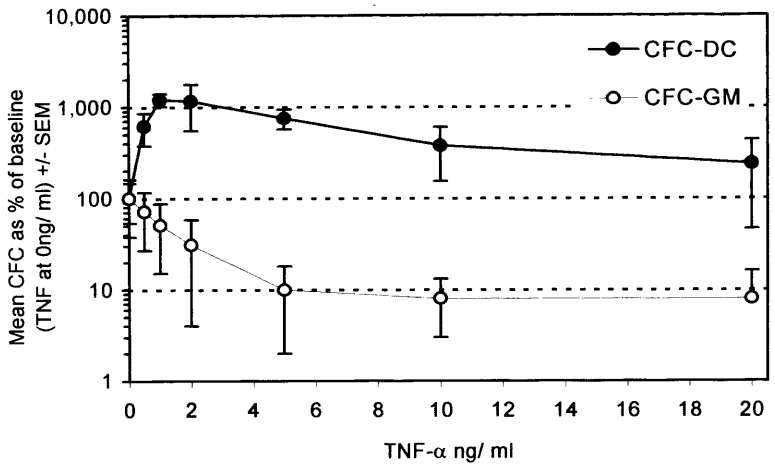
Colony numbers expressed as a % of the baseline number of DC and GM-CFC generated using TNF-α at 0ng/ ml (baseline =100%). Baseline mean ±SEM, CFC-DC = 0.7 ±0.6 and CFC-GM = 30 ±4.7 per $2x10^3$ cells. Mean of 2 separate experiments. Mean purity of CD34$^+$ cells plated was 88% (SEM ±7%).

Fig 3.14 Dose Response of PB CD34$^+$ cells to TNF-α.



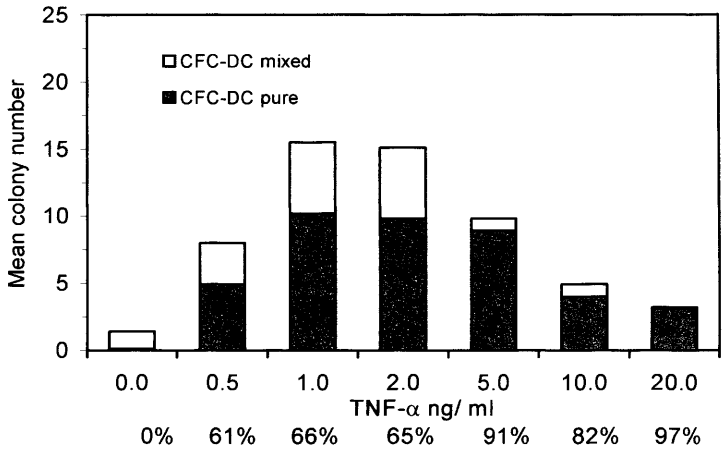
Colony numbers expressed as a % of the baseline number of DC and GM-CFC generated using TNF-α at 0ng/ ml (baseline = 100%). Baseline mean ±SEM, CFC-DC = 1.4 ±1.3 and CFC-GM = 24.7 ±0.6 per $2x10^3$ cells. Mean of 2 separate experiments. Mean purity of CD34$^+$ cells plated was 66% (SEM ±21%).

Fig 3.15a Dose Response of CB CD34$^+$ Cells to TNF-α.



Colony numbers expressed as a % of the baseline number of DC and GM-CFC generated using TNF-α at 0ng/ ml (baseline =100%). Baseline mean ±SEM, CFC-DC = 1.3 ±0.8 and mean CFC-GM = 36 ±16.4 per $2x10^3$ cells. Mean of 3 separate experiments. Mean purity of the CD34$^+$ cells plated was 55% (SEM ±5%).

Fig 3.15b Relative Numbers of Pure and Mixed DC-GM Colonies from CB CD34$^+$ Cells with Increasing Concentrations of TNF-α



The number of pure DC colonies relative to mixed DC-GM colonies with different concentrations of TNF-α used (data derived from the 3 CB samples tested above). Pure DC colonies were almost exclusively seen at TNF-α concentrations of ≥ 5ng/ ml. The % on the x-axis is that of Pure CFC-DC relative to the total CFC-DC numbers.

3.2.2 Dose response experiments: (ii) GM-CSF

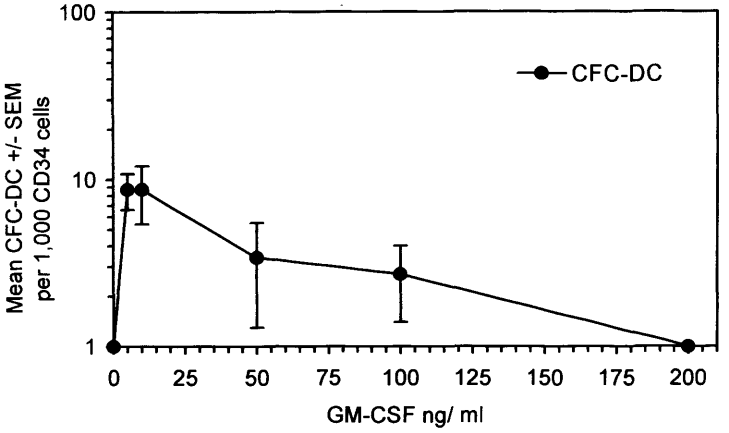
Dose response of $CD34^+$ cells from BM, PB and CB to GM-CSF

Having determined that the best concentration of TNF-α for growth of CFC-DC was 5ng/ ml the next step was to identify the optimum concentration of GM-CSF. In preliminary experiments where $5x10^3$ $CD34^+$ cells from mobilized PB were plated in the CFC-DC assay there was no significant difference between 10 and 100ng/ ml of GM-CSF. The dose response studies however were repeated once it became clear, that the optimum inoculum was 1-2 thousand cells. These experiments, which were conducted, using BM, CB and PB $CD34^+$ cells, demonstrated two important findings:

1. GM-CSF was essential for CFC-DC growth
2. As little as 10ng/ ml of GM-CSF would suffice (Figs 3.16-3.18).

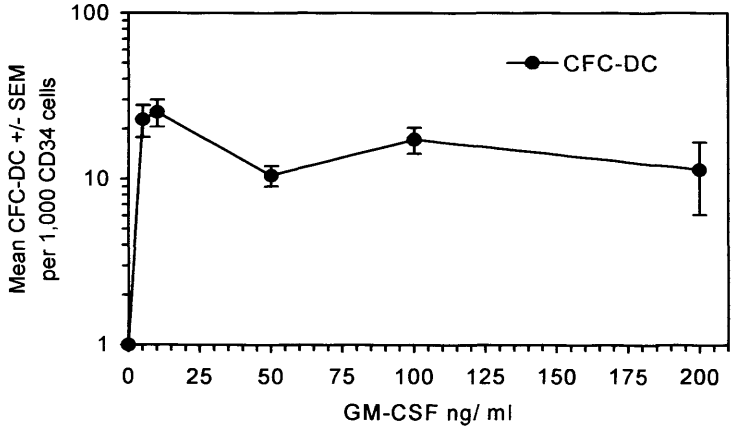
At this low concentration of GM-CSF, CFC-DC growth was superior to higher doses while at the same time a considerable reduction in costs was achieved by using smaller amounts of this cytokine.

Fig 3.16 Dose Response of BM CD34$^+$ cells to GM-CSF.



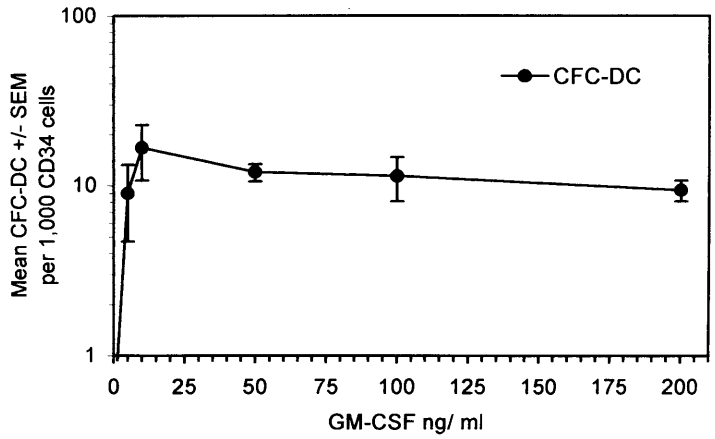
DC colony numbers from BM CD34$^+$ cells generated using different concentrations of GM-CSF (TNF-α used at 5ng/ ml). Mean of 2 separate experiments ±SEM. The purity of the CD34$^+$ cells in the first sample was 74% (purity of the second sample not determined).

Fig 3.17 Dose Response of CB CD34$^+$ cells to GM-CSF.



DC colony numbers generated using different concentrations of GM-CSF (TNF-α used at 5ng/ ml). Mean of 3 separate experiments (n=2 for GM-CSF 50ng/ ml) ± SEM. The purity of one the 3 samples of CD34$^+$ cells post minimacs separation was 71% while that of the remaining 2 samples was not checked.

Fig 3.18 GM-CSF Dose Response of PB CD34$^+$ cells.



DC colony numbers generated using different concentrations of GM-CSF (TNF-α used at 5ng/ ml). Mean of 2 separate experiments ±SEM. Note the similarity with CB CD34$^+$ cells (Fig 3.17). The mean purity (±SEM) of the CD34$^+$ cells plated was 96% ±1.

3.2.3 Morphological confirmation of DC identity

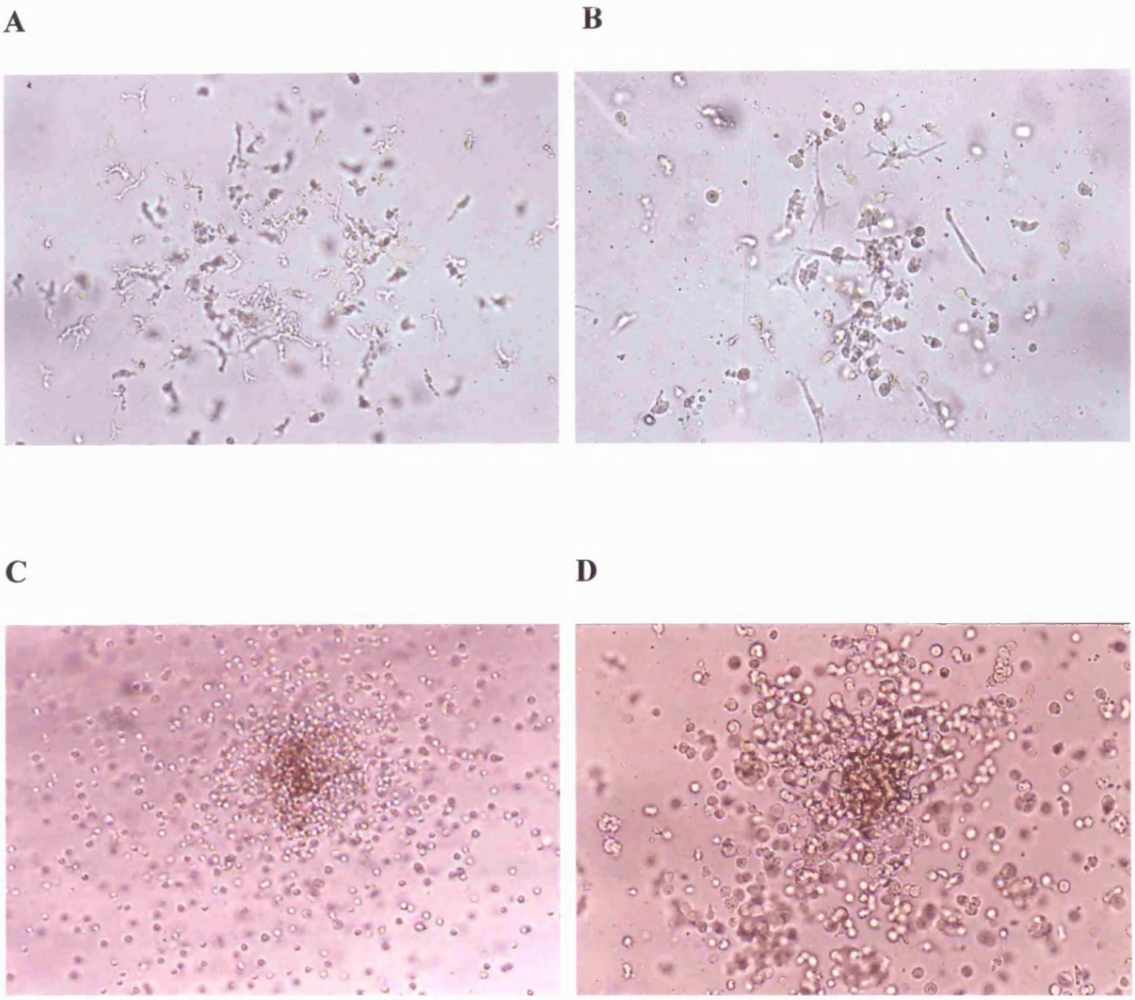
(i) Inverse light microscopy

When $CD34^+$ CB progenitors were cultured in the CFC-DC assay DC colonies developed alongside mixed DC/GM colonies and typical GM colonies. The DC colonies (Fig 3.19a), which consisted almost entirely of DC, were more "spread out" than the relatively compact GM colonies (Fig 3.19d). Each DC colony was made up of cells with long/ short cytoplasmic processes. Some of these cells particularly those with long projections were adherent to the culture dish. The DC exhibited different size/ shape suggesting varying degrees of development/maturation. The mixed DC/ GM colonies contained typical DC, granulocytes and large round macrophages. The CFC type was determined by the predominant cell type in each colony; ie colonies where the majority of cells were Dendritic were scored as pure CFC-DC, colonies where there was a mixture of DC and GM in approximately equal proportions were scored as mixed CFC-DCGM and colonies where the majority of cells were granulocytes and/or macrophages were scored as CFC-GM. Similar morphology was seen when $CD34^+$ cells or MNC from mobilized PB, CB (Fig3.20) or normal BM were plated. The different types of colonies from the CFC-Mix assay are shown in Fig 3.21.

(ii) May-Grunwald Giemsa (MG-G) staining

MG-G staining showed that cells from the DC colonies were large and had folded/ lobulated nuclei and cytoplasmic projections, which varied from short spiky to, elongated veil-like projections (Fig 3.22)

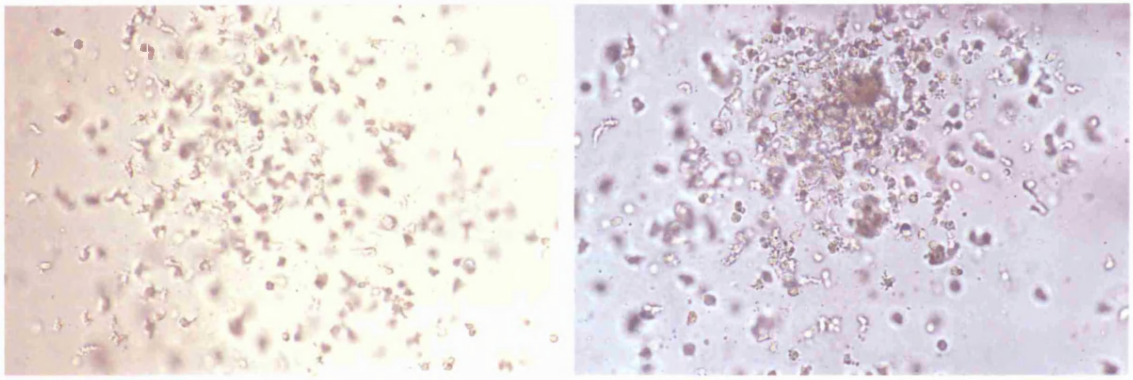
Fig 3.19a-d Morphology of the different colony types from the CFC-DC assay.



CD34^{+} *progenitors* cultured in methylcellulose/IMDM, 30% FCS and GM-CSF/ TNF-α/ SCF yield, pure DC colonies (A), mixed DC/GM colonies (B) and typical granulocyte (C) and GM colonies (D). Granulocyte and GM colonies were scored together. The CD34 cells giving rise to the colonies in A and B were derived from CB and those in C and D from an aphaeresis sample. Original magnification x 125.

Fig 3.20a-b Morphology of Pure DC and Mixed DC/GM Colonies from the CFC-DC assay.

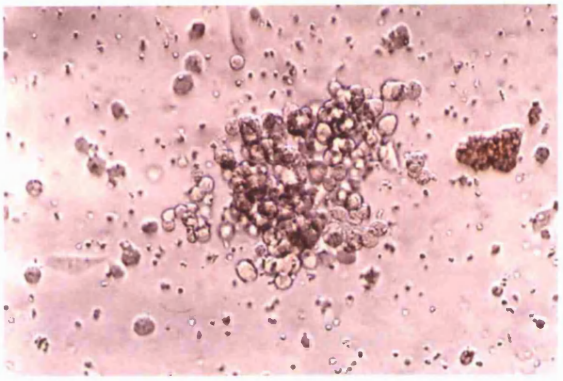
A **B**



Non-fractionated MNC from CB (same sample as that used for Fig 3.19a-b) cultured in methylcellulose/IMDM, 30% FCS and GM-CSF/ TNF-α/ SCF yield pure DC colonies (A) and mixed DC/GM colonies (B). GM colonies similar to those illustrated in Fig 3.19d were also seen. The main difference from plating $CD34^+$ cells was the predominance of the mixed colonies out of the total number of DC colonies. Original magnification x 125.

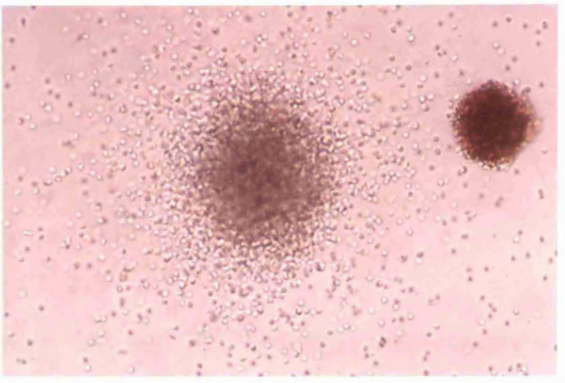
Fig 3.21a-d Morphology of the different colony types from the CFC-Mix assay.

A



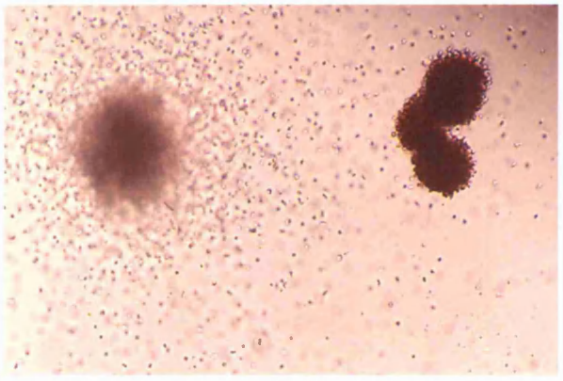
CFC-M

B



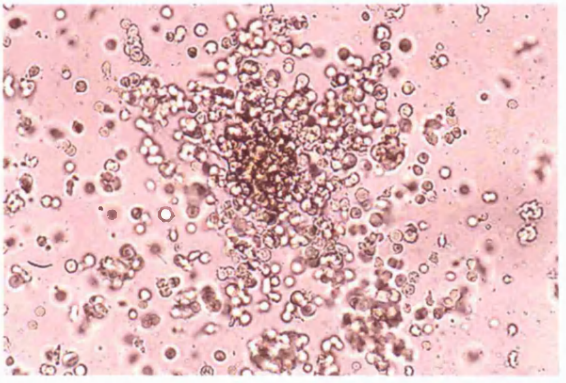
CFC-G and CFU-E

C



CFC-G and BFU-E

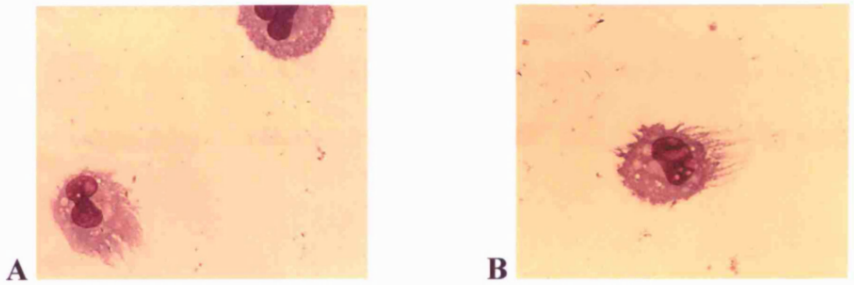
D



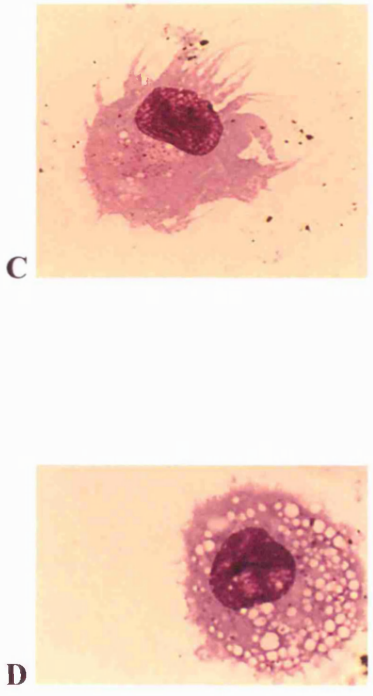
CFC-GM from the DC assay

$CD34^+$ cells from LP cultured in methylcellulose/ IMDM, 30% FCS and Epo yield: (A), typical macrophage colony (CFC-M); (B) granulocyte colony (CFC-G, "red" CFU-E present in the background, top right); (C) BFU-E (3 small "red" colonies, top right) and CFC-G (microscope focused on to the BFU-E). (D) CFC-GM from the *DC assay*. Note the similarity of the CFC-M (A) from the Mix assay with the CFC-GM (D) from the DC assay. Original magnification is x 125 (x 80 for C).

Fig 3.22 May-Grunwald-Giemsa (MG-G) staining of DC.



$CD34^+$ cells from CB cultured in the DC assay. (A) and (B), Cytospin preparations of cells from DC colonies. Original magnification is x600.

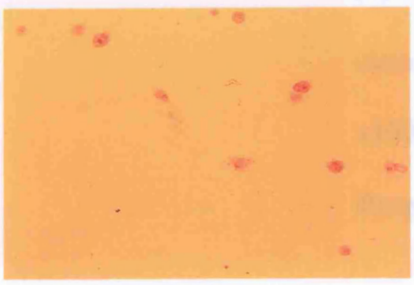


High power views of a Dendritic cell (C) and a Macrophage (D), both from GM colonies grown in the CFC-DC assay (Original magnification x1, 000).

(iii) Immunohistochemistry: CD83, HLA-DR, CD86

The results of immunocytochemical staining are illustrated in Fig 3.23a-c. This showed that cells from the DC colonies were intensely positive for HLA-DP-DR as previously reported (Young et al. 1995). Furthermore the majority of cells from either the pure DC or the mixed DC/GM colonies were positive for CD83 and CD86; two important costimulatory molecules expressed by DC and in particular by mature DC.

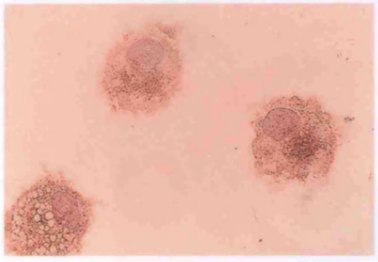
Fig 3.23a (i) – (iv) CD83 Immunophenotype of Pure CFC-DC



(i) CD83$^+$ DC (x100)

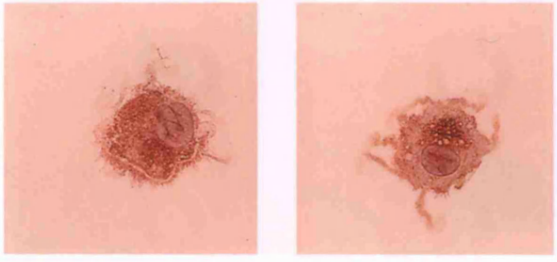
(ii) CD83 control (x100)

BM CD34$^+$ cells cultured in the CFC-DC assay; cells from pure DC colonies stain positive for CD83 (i) (iii) and (iv).



(iii) CD83$^+$ DC (x600)

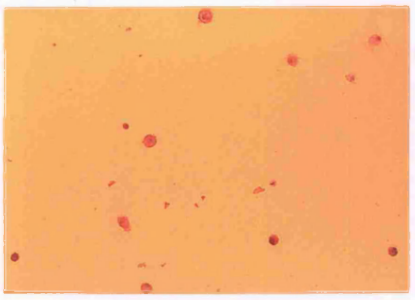
(iii) Mature DC from (i), in high power view stain positive for CD83 (Original magnification x600).



(iv) CD83$^+$ DC (x600)

(iv) Mature DC show strong staining for CD83. CD34$^+$ cells from different BM sample cultured in the DC assay (Original magnification x600).

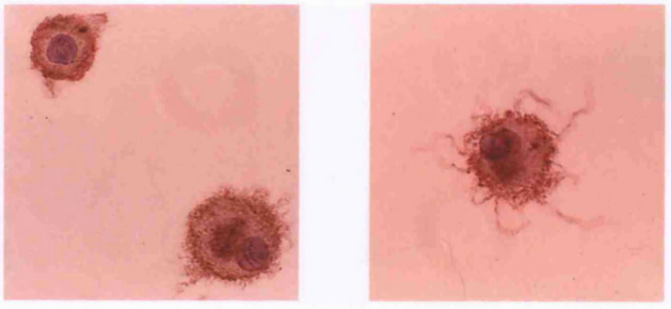
Fig 3.23a (v) – (vii) CD83 Immunophenotype of Mixed CFC-DC/GM



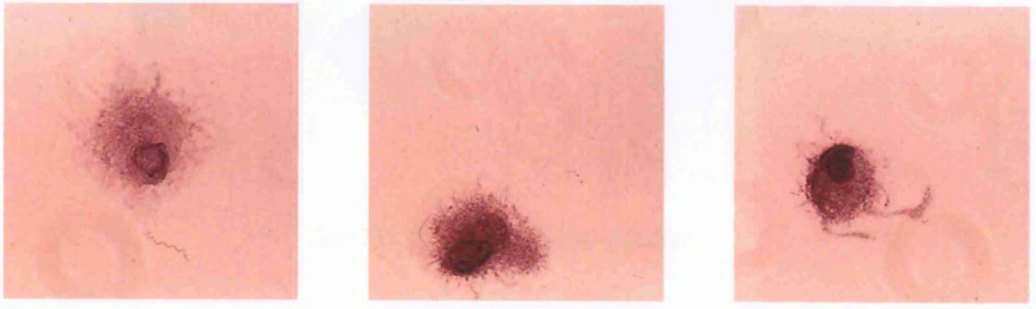
(v) Mature DC present in mixed DC/GM colonies stain positive for CD83; original magnification x100; (vi) DC from (v) in high magnification x600. **Respective controls (vii) show no CD83 staining.**

(v) $CD83^+$ DC (x100)

BM $CD34^+$ cells cultured in the CFC-DC assay for 12-14 days; cells from Mixed DC/GM colonies also stain positive for CD83.



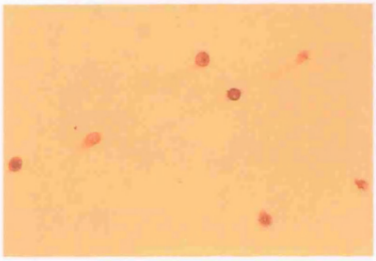
(vi) $CD83^+$ DC (x600)



(vii) $CD83^+$ control (x600)

(vii) The blue-brown cytoplasmic staining is due to the Gill's haematoxylin counterstain

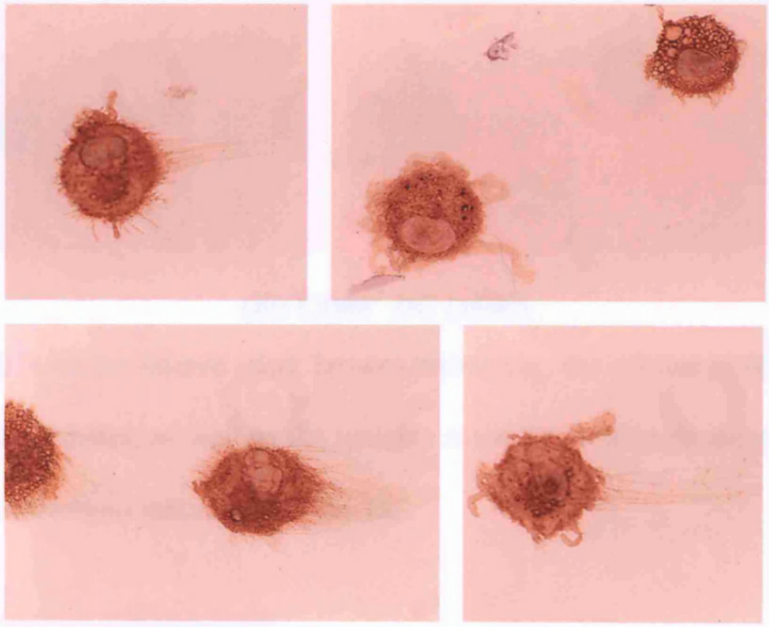
Fig 3.23b (i) – (iv) HLA DP-DR Immunophenotype of Pure CFC-DC



(i) HLA DP-DR$^+$ DC (x100)

(ii) HLA DP-DR control (x100)

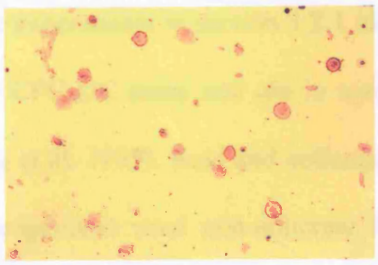
BM CD34$^+$ cells cultured in the CFC-DC assay for 12-14 days; cells from pure DC colonies stain positive for MHC class II antigens (HLA DP-DR) (i) and (iii).



(iii) HLA DP-DR$^+$ DC (x600)

(iii) Note the intense (brown) staining throughout the cells and especially on the plasma membrane; this being consistent with very high expression of MHC class II molecules on mature DC.

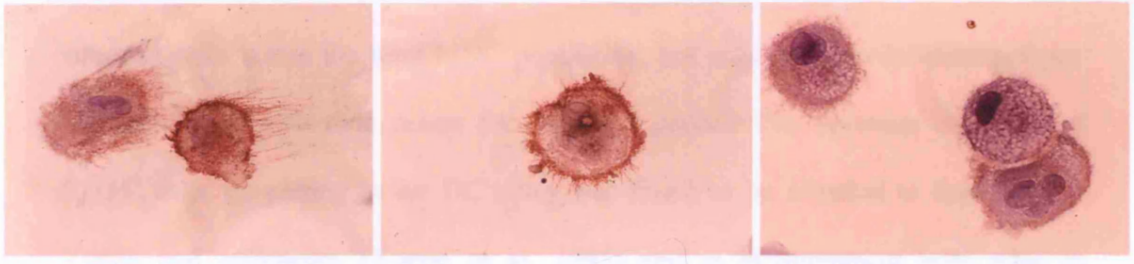
Fig 3.23c (i) – (iv) CD86 Immunophenotype of Pure CFC-DC



(i) CD86$^+$ DC (x100)

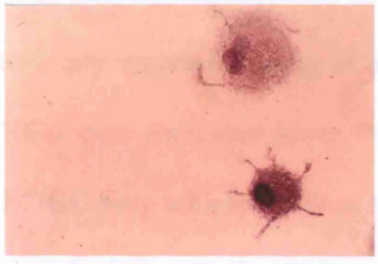
(ii) CD86 control (x100)

BM CD34$^+$ cells cultured in the CFC-DC assay for 12-14 days; cells from pure DC colonies stain positive for CD86 (i) and (iii).



(iii) CD86$^+$ DC (x600)

(iii) Note the intense (dark brown) staining on the plasma membrane and dendrites, as well as the variable intensity of staining suggesting asynchronous maturation of the DC.



(iv) CD86 control (x600)

(iv) The blue-brown cytoplasmic staining is from the counterstain

3.3 Discussion

Linearity

The experiments in section 3.2.1 identified the optimum number of cells for plating in the CFC-DC assay and are in agreement with previous reports (Reid et al. 1990; Young et al. 1995). Reid and colleagues who first developed the clonogenic assay for DC progenitors used non-adherent MNC and showed that linearity was observed when less than $6x10^4$ MNC were plated (Reid et al. 1990). The plating of non-fractionated MNC in this study was preferable particularly for the time course experiments (see chapter 5). This not only allowed comparison with data from the CFC-Mix assay (where non-fractionated MNC are plated routinely) but also avoided an additional variable. This was the anticipated variation in the proportion of non-adherent cells within the total MNC population, not only between different patients but also at different time points for the same patient. The optimum inoculum of $CD34^+$ cells for plating in the DC assay was found to be identical to that used by Young and colleagues (Young et al. 1995) and is in agreement with what is considered to be an appropriate number of cells to plate in other clonogenic assays such as the CFC-Mix (Lord and Marsh 1993).

TNF-α

The optimum concentration of TNF-α was 5ng/ ml, 50% lower than what has been previously reported (Young et al. 1995). Additionally in CB the requirements for TNF-α were somewhat lower than BM or PB CD34 cells, in that peak numbers of CFC-DC were achieved with as little as 1-2ng/ ml. This is in agreement with previous work (Szabolcs et al. 1997), which has shown that in CB HPC, there is some endogenous production of TNF-α or another cytokine/ cytokines acting in a similar

fashion. One possible source of endogenous TNF-α is "contaminating" MNC that would have been present in relatively higher numbers within the immunomagnetically separated $CD34^+$ fraction in CB because of the lower purity post minimacs isolation (55% vs. 88% in BM and 66% in PB). Both T cells and NK cells which would be present within the MNC fraction and are known to inhibit colony formation via production of TNF-α (Trichieri 1992), could secrete this in sufficient quantities to promote CFC-DC growth with very low levels of exogenous TNF-α. Alternatively IL-1β is the other likely candidate because of its TNF-α like effects and the fact that CB $CD34^+$ cells can produce it (Schibler et al. 1994). The TNF-α dose response experiments have demonstrated the following:

1. The importance of TNF-α in the generation of myeloid DC, since in its absence almost no colonies were seen.
2. The bi-directional effect of TNF-α because while it stimulated CFC-DC development at concentrations of 5ng/ ml or less, at the same time it suppressed CFC-GM growth.
3. The relatively inhibitory effect of TNF-α on CFC-DC at concentrations of 10ng/ ml and above observed in BM, CB and mobilized PB.

The first and third findings are supported by published data (Santiago-Schwarz et al. 1993; Santiago-Schwarz et al. 1995). The second finding is supported by previous work that has clearly shown that increasing concentrations of TNF-α at a fixed concentration of GM-CSF and SCF promoted CFC-DC growth while suppressing CFC-GM growth (Saraya and Reid 1995). It contradicts however previous reported data, which showed that the addition of 10ng/ ml TNF-α to 100ng/ ml GM-CSF resulted in no decrease in CFC-GM numbers (Young et al. 1995). Fig 3.13 shows that nearly maximal numbers of CFC-DC was obtained with as little as 0.5ng/ ml of TNF-

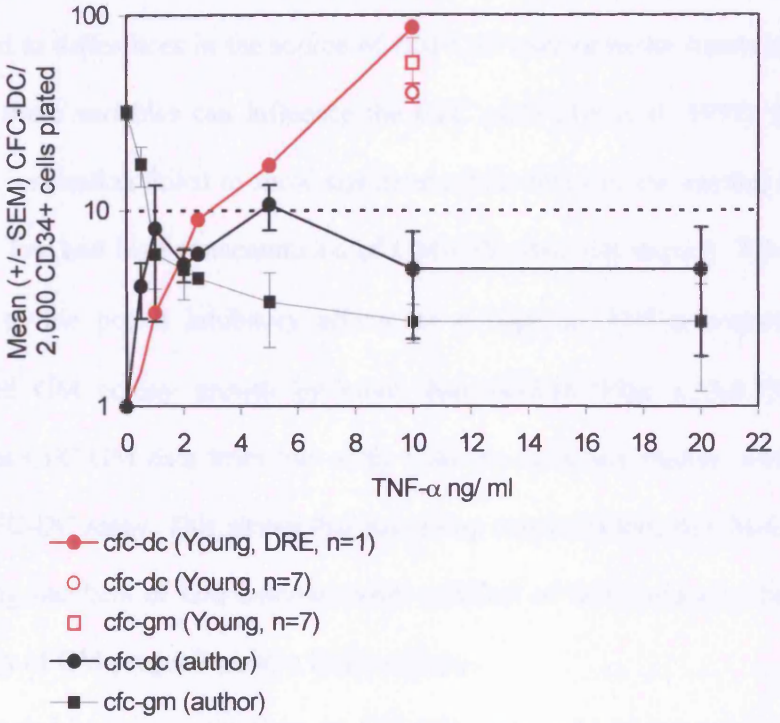
α. At this concentration however CFC-GM numbers were only minimally reduced and a 10-fold decline was not really reached until TNF-α levels increased to ≥2ng/ ml. Similarly the absence of any negative effect on CFC-GM numbers despite the addition of TNF-α to GM-CSF, as observed in Young's study, could be because a "threshold TNF-α level" for inhibition of CFC-GM growth had not been reached and higher concentrations (of TNF-α) were needed to achieve this. This concept is supported by published data. TNF-α is a known suppressor of haemopoiesis *in vitro* (Trichieri 1992) and concentrations as low as 0.1ng/ ml produce 50% inhibition of early CFC-GM (Degliantoni et al. 1985). Another possible contributing factor for the lack of inhibition on CFC-GM growth -despite adding TNF-α and GM-CSF- as observed in the study of Young and co-workers (Young et al. 1995), is the 10-fold higher concentration of GM-CSF used. This can be tested experimentally.

Fig 3.24 shows the effect on CFC-DC and CFC-GM growth from BM $CD34^+$ cells with varying concentrations of TNF-α. Possible explanations for the difference in CFC-DC numbers between this study and that reported by Young and colleagues (Young et al. 1995) where TNF-α was used at 10ng/ ml include the following:

1. Higher inhibitory effect on CFC growth because of a more potent TNF-α batch used by the author.
2. The figures obtained for the dose response evaluation done by Young and co-workers (Young et al. 1995) have been derived from one experiment only and therefore may not be representative of normal BM samples.

It is interesting that in the same study (Young et al. 1995), in a series of seven separate experiments using the same cytokine conditions, the mean number of CFC-DC was 40/ 2,000 $CD34^+$ cells (Fig 3.24). This was much lower than 86 CFC-DC / 2,000 $CD34^+$ cells seen in the one dose response evaluation experiment.

Fig 3.24 Dose Response Evaluation of BM CD34$^+$ Cells to TNF-α and Published data.



The two black lines represent the mean DC and GM colonies from four separate experiments (2 experiments for TNF-α dose levels 0, 0.5 and 1 ng/ ml). These are compared to colony numbers obtained by Young and colleagues (Young et al. 1995) from one dose response evaluation experiment (DRE, red line) and the mean of 7 separate experiments (red circle and square) done using BM CD34$^+$. Cells plated in the same cytokine conditions except for GM-CSF; used at 10ng/ ml by the author and 100ng/ ml by Young and colleagues.

GM-CSF

One surprising finding was that the concentration of GM-CSF needed for optimum CFC-DC growth was considerably lower than previously described. This could be attributed to differences in the source of GM-CSF and/ or in the serum batch used as both of these variables can influence the CFC yield (To et al. 1997). Formal dose response evaluation failed to show significant differences in the number of CFC-GM between low and high concentrations of GM-CSF (data not shown). This is likely to be due to the potent inhibitory effects of ≥ 5ng/ ml TNF-α concentration that decreased GM colony growth by more than 10-fold (Figs 3.13-3.15). Fig 3.25 compares CFC-GM data from this study with two previous studies, which used the same CFC-DC assay. This shows that increasing concentrations of GM-CSF result in increasing numbers of GM colonies while addition of SCF enhances the % cloning efficiency of GM progenitors by a factor of two.

Although higher concentrations of GM-CSF may yield higher numbers of DC in suspension cultures, the number of DC colonies does not necessarily increase with increasing concentrations of GM-CSF. The aim was to choose cytokine conditions, which would result in few GM colonies, as this would facilitate scoring. Moreover GM colonies were assayed using the CFC-Mix assay. Fig 3.26 compares the results of three other studies with those of the author. In particular the data from the study by Ratta and colleagues (Ratta et al. 1998) illustrates this point, as a 5-fold higher concentration of GM-CSF does not seem to yield more CFC-DC.

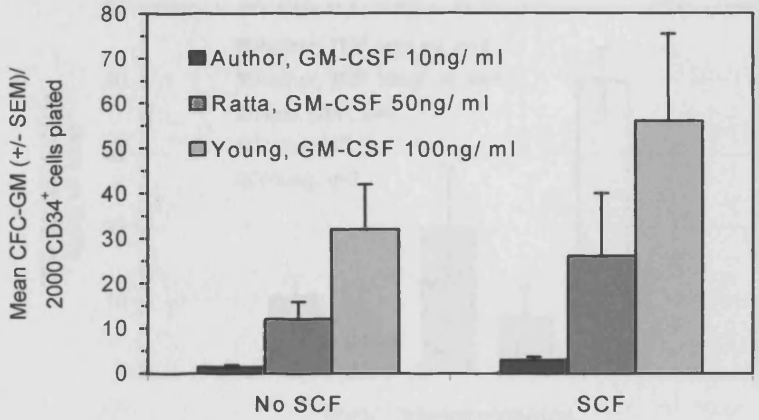
Accurate comparison with the study by Reid and co-workers (Reid et al. 1992) is difficult because they used conditioned medium instead of recombinant growth factors. In addition even though the percentage of FCS used was identical to that used in this study as well as the study by Ratta and colleagues, qualitative differences

between several batches of FCS could account for variations in colony yields (To et al. 1997). Furthermore Reid and co-workers used FACS sorted $CD34^+$ cells which were plated in small numbers (100-1,000) thus minimizing any inhibitory effects by CD34 neg cells. In addition their criteria for scoring CFC-DC (clusters of > 4 cells) were less strict than the criteria employed in this study. Colonies were scored if they consisted of >50 cells (Heyworth and Spooncer 1993). Fig 3.26 shows that the number of CFC-DC in BM was comparable to that reported by two other independent studies and only the data from Young's group showed a significantly higher frequency of CFC-DC. Other possible explanations for this discrepancy are:

- The use of 20%FCS by Young and co-workers instead of 30%
- The use of agarose as a semi-solid medium instead of methylcellulose

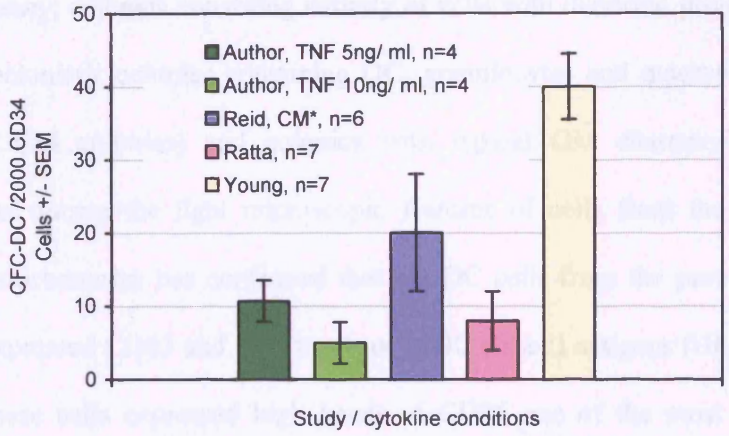
It is possible that the lower percentage of FCS in the culture medium may have reduced any potential "growth-suppressing" effects from inhibitory cytokines in the serum or that the FCS used by Young and colleagues was an exceptionally good batch for DC growth.

Fig 3.25 Comparison of CFC-GM numbers, generated with and without 20ng/ ml SCF, with two previous studies.



BM $CD34^+$ cells plated in the CFC-DC assay with 10ng/ ml TNF-α and the following GM-CSF concentrations: 10ng/ ml GM-CSF (n=4, author), 50ng/ ml GM-CSF (n=7) (Ratta et al. 1998) or 100ng/ ml GM-CSF (no SCF, n=6; +SCF, n=7) (Young et al. 1995). The data for the "no SCF" group for GM-CSF 10ng/ ml concentration have been calculated based on the fact that SCF enhances CFC-GM growth by a factor of ~2 (Ratta et al. 1998; Young et al. 1995).

Fig 3.26 Comparison of CFC-DC numbers generated by plating BM CD34$^+$ cells in different culture conditions.



Author, 30% FCS, TNF-α 5 or 10 ng/ ml plus GM-CSF 10ng/ ml, SCF 20ng/ ml in methylcellulose; Reid, 30% FCS, leucocyte-conditioned medium, FACS sorted CD34$^+$ cells; Ratta, 30% FCS, TNF-α 10 ng/ ml, GM-CSF 50 ng/ ml, SCF 20 ng/ ml in methylcellulose; Young, 20% FCS, TNF-α 10ng/ ml, GM-CSF 100 ng/ ml, SCF 20 ng/ ml in agarose.

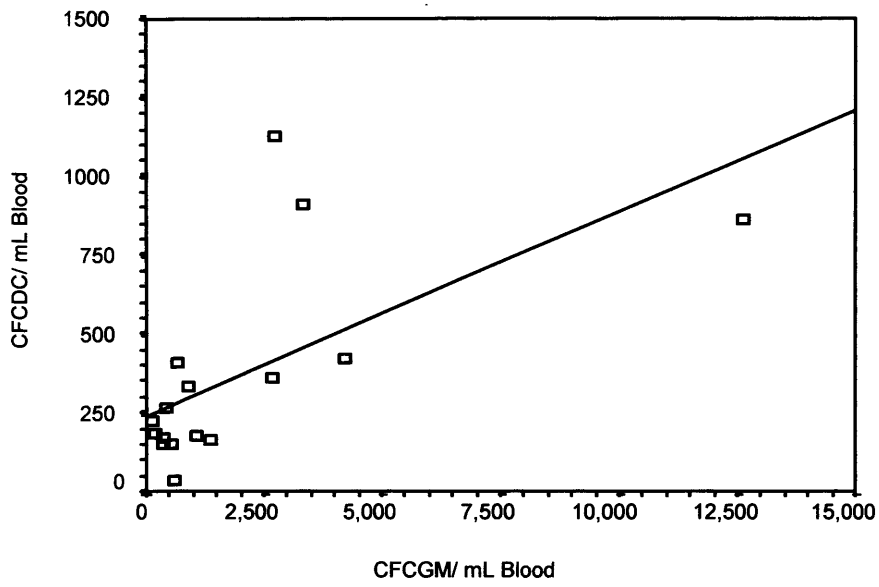
Morphological confirmation

Inverse Light microscopy showed the development of 3 types of colonies in the CFC-DC assay; colonies consisting entirely of cells with dendritic projections (called pure DC colonies), colonies containing DC, granulocytes and macrophages (termed mixed DC/GM colonies) and colonies with typical GM characteristics. Giemsa staining has shown the light microscopic features of cells from the DC colonies. Immunohistochemistry has confirmed that the DC cells from the pure or mixed DC colonies expressed CD83 and high levels of MHC class II antigens (HLA-DP-DR). In addition these cells expressed high levels of CD86 one of the most important co-stimulatory molecules involved in the priming of T cells (Viola et al. 1999). Expression of MHC class II and CD86 implies that the DC obtained from the colonies ought to be capable of stimulating allogeneic $CD4^+$ T-helper cells *in vivo* or *in vitro* and this demonstrates that quantification of CFC-DC measures true progenitors of putative functional DC.

Correlation of CFC-DC with CFC-GM

The numbers of mobilized circulating CFC-DC in patients correlated positively with the numbers of CFC-GM determined using the DC and Mix assays respectively (Fig 3.27). Myeloid DC and GM, share a common progenitor (CFC-GMDC) (Young 1999b). Therefore the strong positive correlation obtained by measuring CFC-DC and CFC-GM in separate assays adds more to the validity of the DC assay.

Fig 3.27 Correlation between CFC-DC and CFC-GM Numbers.



Positive correlation between CFC-DC and CFC-GM numbers from mobilized PB in 13 patients with solid tumours (see chapter 5) collected on the day of harvest (n=17, four patients had 2 aphereses). CFC-DC and CFC-GM have been determined by plating $CD34^+$ cells in the DC assay and the Mix assay respectively. Pearson correlation coefficient, $r = 0.634$ ($p = 0.006$).

3.4 Conclusions

The following conclusions can be drawn so far:

- The optimum number of cells that should be plated in the CFC-DC assay was 5x 10^4 MNC and 0.5-2x 10^3 $CD34^+$ cells (1,000-1,500 cells probably preferable to 2,000). The same applied to $AC133^+$ cells.
- The optimum concentration of TNF-α was 5ng/ ml although this can depend on the batch used and therefore a new batch should always be tested. As far as GM-CSF is concerned it appears that low concentrations (10ng/ ml) were sufficient.

Further work is needed to establish if any as yet unidentified cytokines exist, which stimulate CFC-DC growth *in vitro* or if there is a *"DC-CSF"*. FLT-3L has been shown to enhance DC growth *in vivo*. Daily treatment of mice resulted in huge increases of DC in lymphoid organs, BM and PB (Maraskovsky et al. 1997; Shurin et al. 1997). Similar findings have been found in humans (Maraskovsky et al. 2000). However although FLT-3L enhances DC production by expansion of progenitor cells, one study (Ratta et al. 1998) has failed to show synergy *in vitro* with other cytokines like GM-CSF/ TNF-α/ SCF which are important for the generation of myeloid DC. Recent work has shown that FLT-3L (and SCF) does not affect DC differentiation but instead it maintains the expansion of CFC-DC, which in the presence of GM-CSF, +TNF-α produces more mature DC (Curti et al. 2001). This would suggest that there is possibly an as yet unidentified cytokine/s which act/s synergistically with GM-CSF+TNF-α and exerts growth/ differentiation promoting effects at the level of the committed clonogenic progenitor. It is therefore imperative that further work in this field continues because identification of such a growth factor could have an impact in the therapeutics of cancer, immunosuppression, and infectious diseases and transplant rejection.

Chapter 4

A Comparative Study of DC Progenitors in BM, CB, Mobilized PB and AC133 and CD33 Expression on $CD34^+$ Progenitors

4.1 Introduction

Yin and colleagues have demonstrated that HPC expressing the novel stem cell antigen AC133 contain the majority of cells committed to the GM pathway (Yin et al. 1997). Myeloid (as supposed to lymphoid) DC develop from myeloid lineage progenitors expressing markers like CD13 and CD33 (Reid 1997). It was therefore hypothesized that $CD34^+$ cells co-expressing the AC133 antigen should contain the majority of myeloid DC progenitors. To test this, immunomagnetically separated $CD34^+$ cells from BM, CB and mobilized PB (including LP) were sorted into AC133 positive and negative fractions using a FACS Vantage (please see Methods, chapter 2, section 2.3.4). The 2 cell fractions obtained, $CD34^+AC133^+$ and $CD34^+$ $AC133^-$ were then plated in the CFC-DC and CFC-Mix assays. The expression of the myeloid antigen CD33 was similarly assessed and for some samples this was tested together with AC133 expression. This was done to establish which of the 2 antigens was preferentially expressed on myeloid DC progenitors.

Additionally the frequency CFC-DC in $CD34^+$ cells from BM, CB and mobilized PB was examined in a large number of samples. Immunomagnetically separated $CD34^+$ cells were plated under identical culture conditions, in the CFC-DC and CFC-Mix assays in order to quantify DC progenitors. This would help identify the best source of $CD34^+$ cells containing committed DC progenitors for *ex vivo* expansion.

4.2 Results

4.2.1 AC133 expression of human $CD34^+$ cells from BM, CB, and mobilized PB

The proportion of $AC133^+$ cells within the $CD34^+$ population was measured in a large number of samples (Fig 4.1). The results demonstrated considerable differences between BM, CB and mobilized PB in the proportion of $AC133^+$ cells among the $CD34^+$ population. The corresponding median values (range) were: 36% (29-74%) in BM, 63% (41-87%) in CB, 78% (65-100%) in mobilized PB and 78% (61-84%) in LP. Fig 4.1 shows that the greatest variation was seen in BM and CB while in mobilized PB/ LP there was less variation between different samples.

4.2.2 Frequency of CFC in the $AC133^{+/-}$ and $CD33^{+/-}$ fractions of $CD34^+$ cells

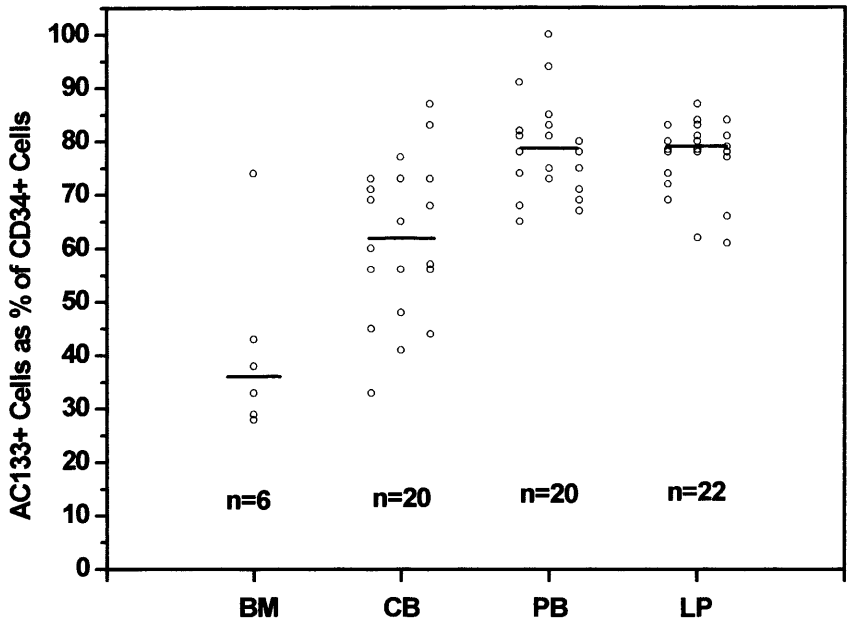
(i) Bone marrow

The frequency of CFC-DC and CFC-GM was higher in $CD34^+AC133^+$ cells (13 CFC-DC and 23 CFC-GM per 1,000 cells) than $CD34^+AC133^-$ cells (2 CFC-DC and 7 CFC-GM per 1,000 cells) while that of BFU-E was higher in single positive cells (45 versus 10 per 1,000 cells) (Fig 4.2a). $CD34^+$ progenitors co-expressing CD33 had a slightly higher frequency of CFC-DC (12 per 1,000 cells) and a 23-fold higher frequency of GM (31 per 1,000 cells) than $CD34^+CD33^-$ cells (8 and 1 per 1,000 cells respectively- frequencies have been rounded off to the nearest decimal point) (Fig 4.2b). BFU-E had the same frequency in both $CD33^+$ and $CD33^-$ fractions (54 and 56 per 1,000 cells respectively).

(ii) Cord blood

The frequencies of CFC-DC and CFC-GM were higher in the $AC133^+$ fraction; 22 and 6 per 1,000 $CD34^+AC133^+$ cells versus 2 and 2 respectively per 1,000 $CD34^+AC133^-$ cells (Fig 4.3b).

Fig 4.1 Expression of the AC133 Antigen on $CD34^+$ cells



The % of $AC133^+$ cells within the $CD34^+$ cell population = (% $AC133^+$ cells in sample/ % $CD34^+$ cells in same sample) x100. There were no $AC133^+$ cells, which were $CD34^-$ in accordance with previously published data (de Wynter et al. 1998; Pasino et al. 2000). The PB and LP samples were obtained from the same patients on the day of harvest. Two of the 22 patients did not have PB analysis. The horizontal bars denote the median of n individual determinations. The differences between the medians (CB versus BM/ PB) were significant (Kruskall-Wallis test, $\chi^2(2) = 10.8$, $p = 0.005$).

The frequency of BFU-E was much higher in $CD34^+AC133^-$ than $CD34^+AC133^+$ cells (265 versus 94 per 1,000 cells respectively). The frequencies of CFC-DC/ GM in CB $CD34^+CD33^+$ cells were 11 and 8 respectively per 1,000 cells compared to 7 CFC-DC and 2 CFC-GM per 1,000 $CD34^+CD33^-$ cells (Fig 4.3b). BFU-E had a higher frequency in the single positive fraction ($CD34^+CD33^-$), 210 versus 134 per 1,000 cells respectively.

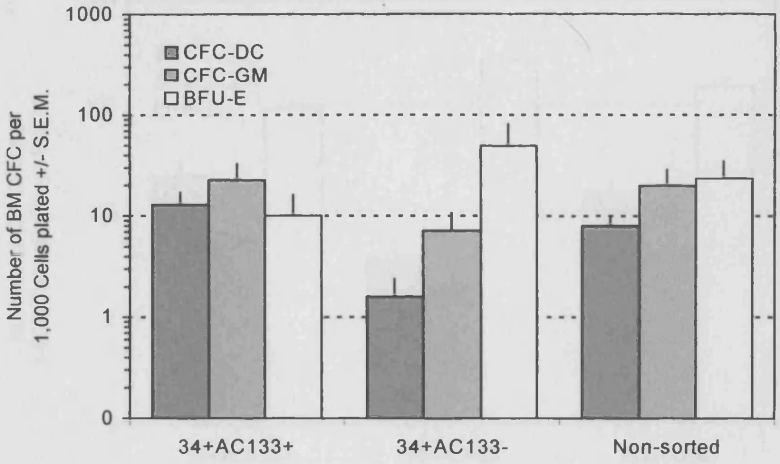
(iii) Mobilized peripheral blood

This was similar to BM apart from one striking observation (Fig 4.4a); the difference in the respective frequencies of CFC-DC and CFC-GM, between $CD34^+AC133^+$ and $CD34^+AC133^-$ cells was greater in mobilized PB than BM. There were 15 CFC-DC and 33 CFC-GM per 1,000 $CD34^+AC133^+$ cells versus <1 CFC-DC and 1 CFC-GM per 1,000 $CD34^+AC133^-$ cells. CFC-DC were not detected in the $CD34^+AC133^-$ fraction in 3/3 PB samples tested despite plating 1,000 and 2,000 cells in the DC assay. The frequency of BFU-E was 6-fold higher in $AC133^-$ cells than $AC133^+$ cells. For cells sorted on the basis of CD33 expression (Fig 4.4b), the frequencies of CFC-DC were comparable for double and single cells (8 versus 7 respectively per 1,000 cells) and of CFC-GM were 2-fold higher in the double positive fraction (41 versus 22 per 1,000 cells). The frequency of BFU-E was higher in the single positive fraction (106 versus 39 per 1,000 cells).

(iv) Leucaphaeresis products

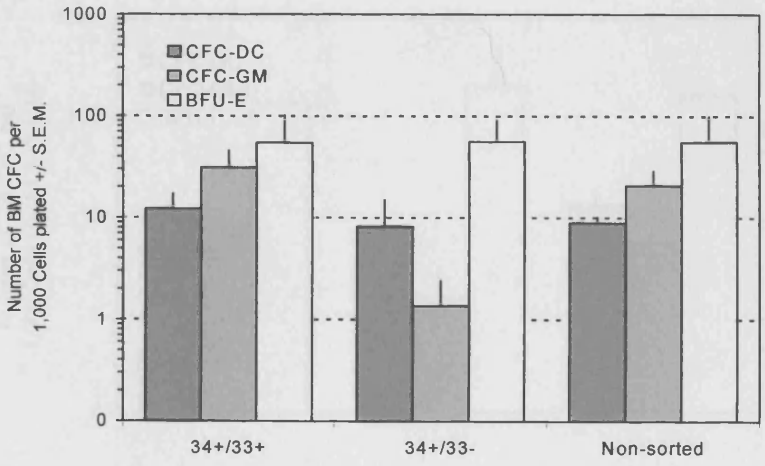
In 5 LP examined where the progenitors were sorted on the basis of AC133 (Fig 4.5a) and CD33 expression (Fig 4.5b), the patterns were broadly similar to PB (Figs 4.4a and 4.4b). However for cells sorted by AC133 expression, strict comparison with PB samples is not possible, as of the 5 patients who had their aphaeresis samples tested, only 3 had PB samples analysed simultaneously

Fig 4.2a AC133 Expression and CFC Numbers in BM



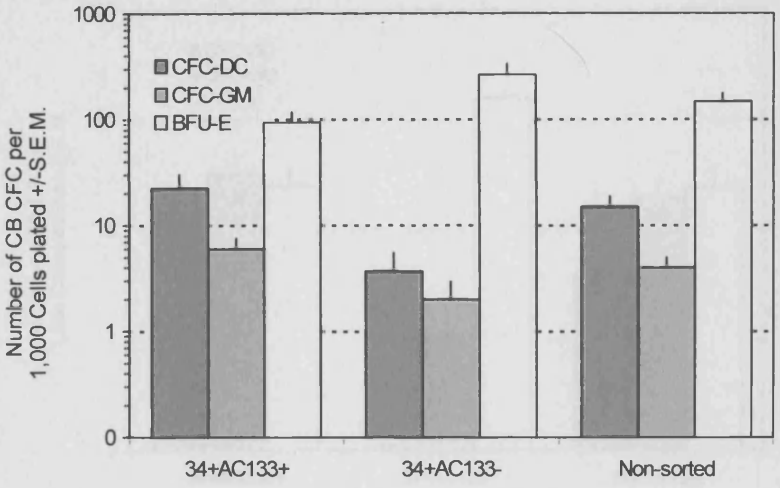
$CD34^+$ cells (mean purity, 73% ±11 SEM) were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-DC assay, for DC Progenitors and CFC-Mix assay, for GM and Erythroid Progenitors (n=3).

Fig 4.2b CD33 Expression and CFC Numbers in BM



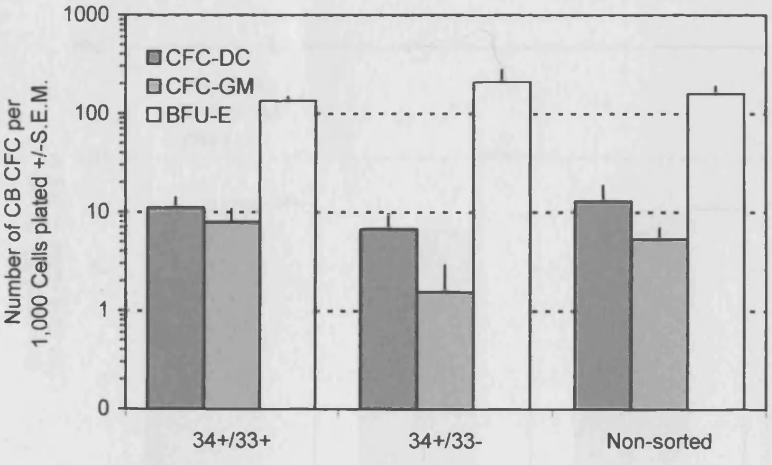
$CD34^+$ cells (mean purity, 70% ±9 SEM) were sorted into $CD33^+$ and $CD33^-$ fractions and plated in the CFC-DC and CFC-Mix assays (n=3, two of the three samples were the same, as the ones tested above for AC133 expression).

Fig 4.3a AC133 Expression and CFC Numbers in CB



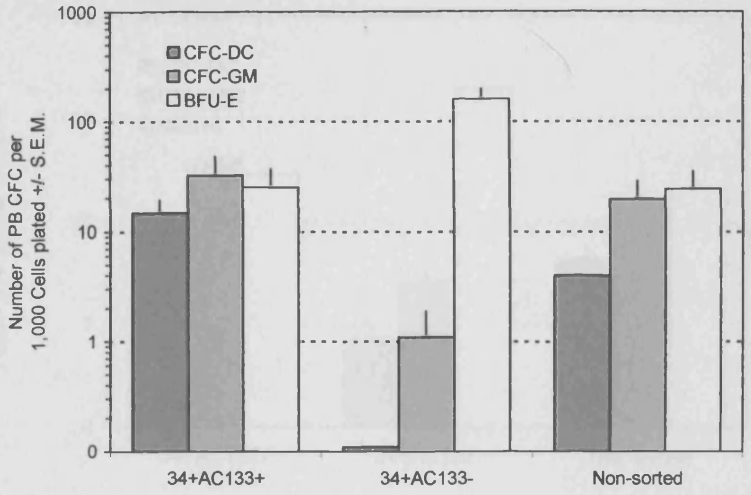
$CD34^+$ cells (mean purity, 66% ±8 SEM) were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-DC assay, for DC Progenitors and CFC-Mix assay, for GM and Erythroid Progenitors (n=5).

Fig 4.3b CD33 Expression and CFC Numbers in CB



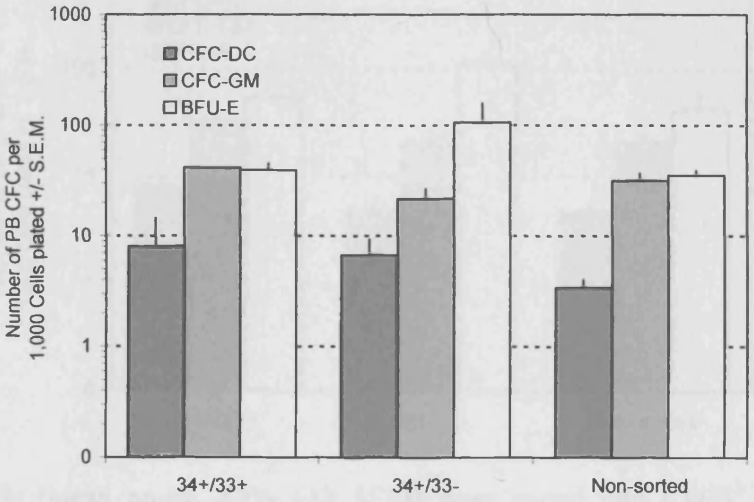
$CD34^+$ cells (mean purity, 69% ±9 SEM) were sorted into $CD33^+$ and $CD33^-$ fractions and plated in the CFC-DC and CFC-Mix assays (n=4, only two of the four samples were the same as the ones tested above for AC133 expression).

Fig 4.4a AC133 Expression and CFC Numbers in Mobilized PB



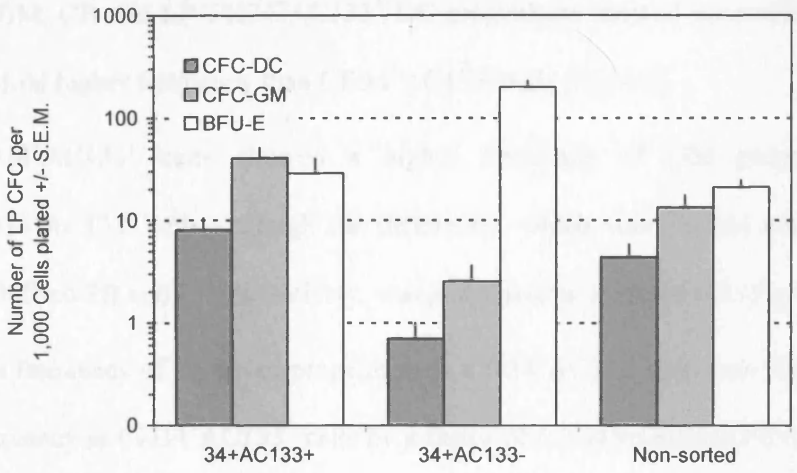
$CD34^+$ cells (mean purity, 77% ±23 SEM) were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-DC assay, for DC Progenitors and CFC-Mix assay, for GM and Erythroid Progenitors (n=3). No CFC-DC were obtained despite plating 1,000 and 2,000 $CD34^+AC133^-$ cells; the short bar has been inserted to show that $CD34^+AC133^+$ cells were plated in the DC assay.

Fig 4.4b CD33 Expression and CFC Numbers in Mobilized PB



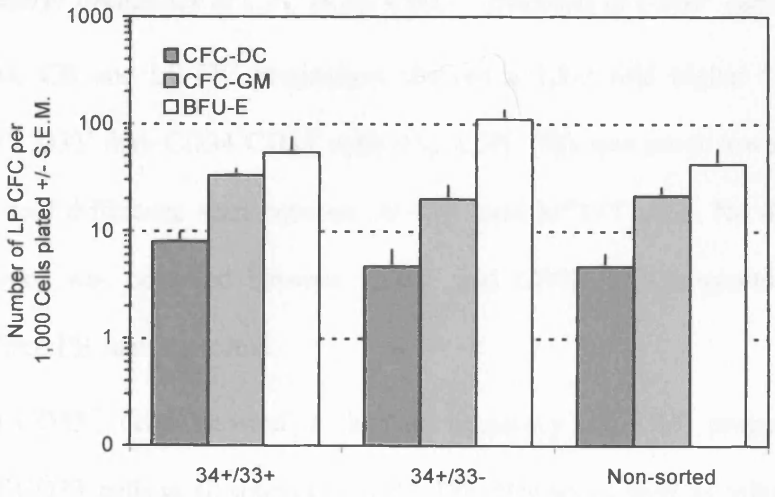
$CD34^+$ cells (mean purity, 96% ±1 SEM) were sorted into $CD33^+$ and $CD33^-$ fractions and plated in the CFC-DC and CFC-Mix assays (n=2, the two samples were the same as 2 of the 3 samples tested above for AC133 expression).

Fig 4.5a AC133 Expression and CFC Numbers in LP



$CD34^+$ cells (mean purity, 64% ±10 SEM) were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-DC assay for DC Progenitors and CFC-Mix assay, for GM and Erythroid Progenitors (n=5).

Fig 4.5b CD33 Expression and CFC Numbers in LP



$CD34^+$ cells (mean purity, 69% ±12 SEM) were sorted into $CD33^+$ and $CD33^-$ fractions and plated in the CFC-DC and CFC-Mix assays (n=4, all samples were the same as 4 out of 5 samples used above for AC133 sorting).

(v) Summary: Frequency of CFC in the AC133$^{+/-}$ fractions of CD34^{+} cells

- In BM, CB and LP CD34^{+}AC133^{+} DC progenitors showed approximately a 6 to 12-fold higher frequency than CD34^{+}AC133^{-} cells (Fig 4.6).
- CD34^{+}AC133^{+} cells showed a higher frequency of GM progenitors than CD34^{+}AC133^{-} cells although the difference, which was 29-fold and 16-fold in mobilized PB and LP respectively, was just 3-fold in BM and CB (Fig 4.7)
- The frequency of erythroid progenitors in CD34^{+}AC133^{-} cells was higher than the frequency in CD34^{+}AC133^{+} cells by a factor of 6-7 in mobilized PB and LP and a factor of 3-5 in CB and BM (Fig 4.8).
- The frequency ratios of GM to DC progenitors (non-sorted CD34^{+} cells) were higher in BM (2.5:1), mobilized PB (4.9:1) and LP (3:1). This trend was reversed in CB where the GM to DC progenitor ratio was 1:3.7.

(vi) Summary: Frequency of CFC in the CD33$^{+/-}$ fractions of CD34^{+} cells

- In BM, CB and LP DC progenitors showed a 1.5-2 fold higher frequency in CD34^{+}CD33^{+} than CD34^{+}CD33^{-} cells (Fig 4.10). This was much lower than the 6 to 12-fold difference seen between AC133^{+} and AC133^{-} cells. No difference in frequency was observed between CD33^{+} and CD33^{-} DC progenitors in the 2 mobilized PB samples tested.
- CD34^{+}CD33^{+} cells showed a higher frequency of GM progenitors than CD34^{+}CD33^{-} cells in all sources of HPC. The differences were as follows: 23-fold in BM, 5-fold in CB, 2-fold in mobilized PB and nearly 2-fold in LP.
- A proper comparison of frequency between CD33$^{+/-}$ cells and AC133$^{+/-}$ cells would not be valid because only 2/3 BM samples, 2/5 CB samples and 4/5 LP

samples tested by FACS sort analysis were the same. However the frequencies of CFC in $AC133^{+/-}$ and $CD33^{+/-}$ cells were compared in 4 LP. $CD34^{+}AC133^{+}$ cells had an 8.1 and 15.4-fold higher frequency than $CD34^{+}AC133^{-}$ cells for CFC-DC and CFC-GM respectively. In marked contrast $CD34^{+}CD33^{+}$ cells showed only a 1.7 and 1.6-fold higher frequency than $CD34^{+}CD33^{-}$ cells for CFC-DC and CFC-GM respectively. A comparison of the mean fold difference (Wilcoxon signed-rank test) between $AC133^{+/-}$ cells with that observed in $CD33^{+/-}$ cells in the 4 LP tested, showed a significant difference for CFC-GM (19.5-fold vs. 2.7-fold in the AC133 vs. CD33 groups respectively; one tailed p value = 0.034) and borderline significant for CFC-DC (6-fold vs. 1.7-fold in the AC133 vs. CD33 groups respectively; one tailed p value = 0.055).

- In BM the frequency of BFU-E was comparable in $CD33^{+}$ and $CD33^{-}$ cells. In CB, mobilized PB and LP the frequency of BFU-E was 2 to 3 times greater in $CD33^{-}$ progenitors (Fig 4.12).

4.2.3 Total numbers of CFC in single and double positive cells

The numbers of CFC in the CD34 fractions have been calculated taking into account the differences in frequency of the progenitors and the relative fractions of $AC133^{+}$ versus $AC133^{-}$ cells and $CD33^{+}$ versus $CD33^{-}$ cells.

Total cell numbers and AC133 expression

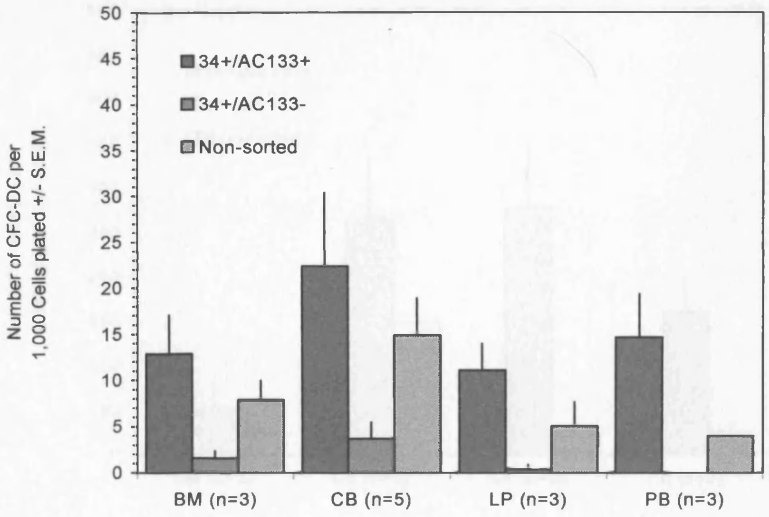
The total numbers of CFC-DC and CFC-GM were higher in the AC133 positive fraction in all sources of HPC tested (Table 4.1). Figs 4.6-4.8 summarize a comparison of the frequencies of CFC-DC, CFC-GM and BFU-E obtained from $CD34^{+}$ cells sorted on the basis of AC133 expression in BM, CB and mobilized PB.

Table 4.1 Total CFC Numbers per 1,000 $CD34^+$ cells (AC133 Antigen)

	CFC from $CD34^+AC133^+$ fraction	CFC from $CD34^+AC133^-$ fraction	Fold difference[†]	P value
BM (n=3)				
CFC-DC	6.6 ±2.2	0.6 ±0.4	**x10**	**0.05**
CFC-GM	12.0 ±6.0	3.0 ±1.6	x4	ns
BFU-E	5.8 ±2.5	17.7 ±8.0	x0.3	ns
CB (n=5)				
CFC-DC	16.9 ±6.1	0.9 ±0.5	**x19**	**0.009**
CFC-GM	4.4 ±1.0	0.6 ±0.3	**x7**	**0.009**
BFU-E	69.3 ±15.9	66.3 ±16.8	x1	ns
PB (n=3)				
CFC-DC	13.2 ±4.4	0	**(NE[‡])**	**0.035**
CFC-GM	28.6 ±14.3	0.1 ±0.1	**x286**	**0.05**
BFU-E	22.2 ±10.5	14.6 ±2.5	x2	ns
LP (n=3)	Same samples as 3 PB samples above			
CFC-DC	9.7 ± 2.9	0.01 ±0	**x970**	**0.046**
CFC-GM	24.0 ±10.6	0.4 ±0.4	**x60**	**0.046**
BFU-E	25.2 ±9.2	26.3 ±18.1	x1	ns
LP (n=5)				
CFC-DC	6.9 ±2.3	0.1 ±0	**x63**	**0.008**
CFC-GM	31.3 ±8.7	0.6 ±0.3	**x52**	**0.009**
BFU-E	23.3 ±7.6	34.5 ±11.1	x1	ns

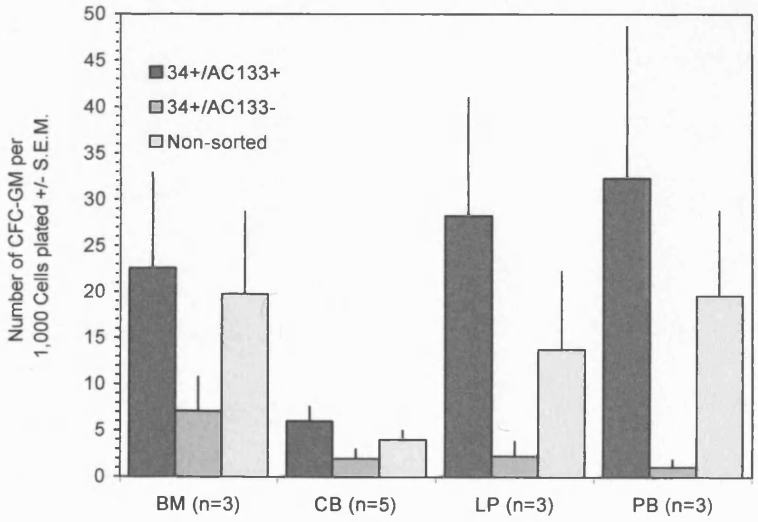
[†] Fold difference compared to single positive cells; Fold differences have been approximated to the nearest decimal point; [‡] NE, not evaluable as denominator (CFC-DC in $CD34^+AC133^-$ cells) was 0. CFC-GM data were derived from the CFC-mix assay; p values refer to comparison of total CFC-DC and CFC-GM numbers in $CD34^+$ cells derived from the $AC133^+$ and $AC133^-$ fractions (Mann-Whitney test); ns = not significant.

Fig 4.6 Frequency of CFC-DC in BM, CB, LP and Mobilized PB



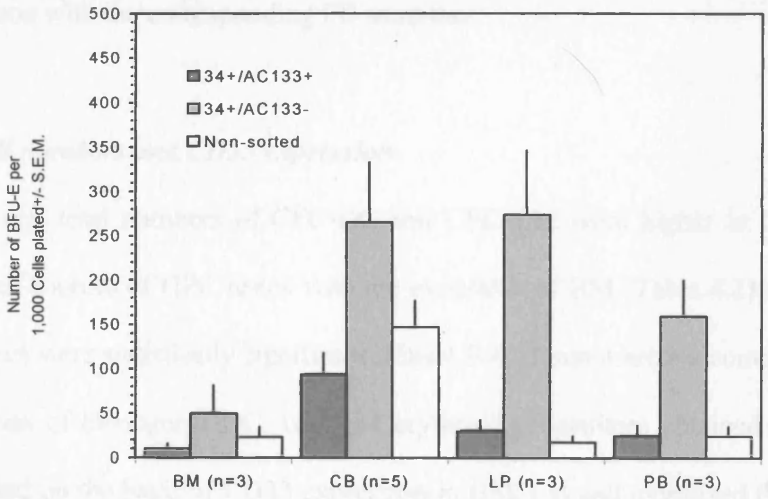
$CD34^+$ cells were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-DC assay. LP and PB samples were from the same patients.

Fig 4.7 Frequency of CFC-GM in BM, CB, LP and Mobilized PB



$CD34^+$ cells were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-Mix assay. LP and PB samples were from the same patients.

Fig 4.8 Frequency of BFU-E in BM, CB, LP and Mobilized PB



$CD34^+$ cells were sorted into $AC133^+$ and $AC133^-$ fractions and plated in the CFC-Mix assay. LP and PB samples were from the same patients.

Only data for 3 out of the 5 LP samples tested are displayed in figs 4.6-4.8 to enable a comparison with the corresponding PB samples.

Total cell numbers and CD33 expression

Although total numbers of CFC-DC and CFC-GM were higher in $CD34^+CD33^+$ cells in all sources of HPC tested with the exception of BM (Table 4.2) none of these differences were statistically significant. Figs 4.9-4.11 summarize a comparison of the frequencies of clonogenic DC, GM and erythroid progenitors obtained from $CD34^+$ cells sorted on the basis of CD33 expression in BM, CB and mobilized PB. Only data for 2 out of the 4 LP samples tested are displayed in figs 4.9-4.11 to enable a comparison with the corresponding PB samples.

4.2.4 Frequency of $CD34^+$ DC progenitors in BM, CB, PB and LP

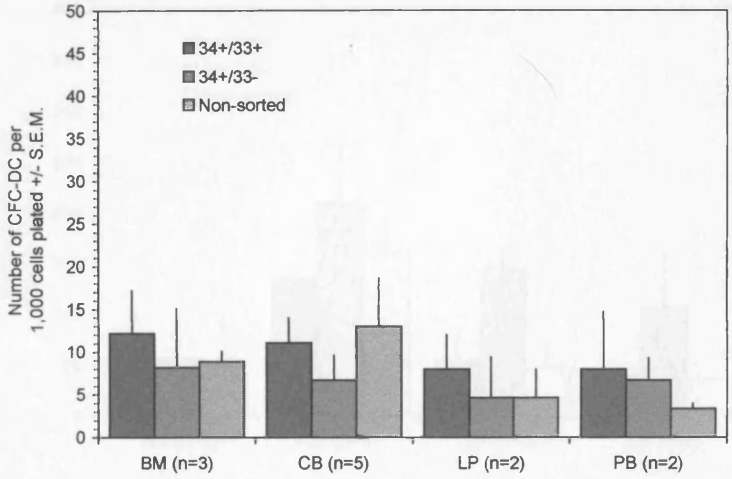
Fig 4.12 shows the colony numbers obtained in all the clinically important sources of HPC. The frequency of CFC-DC was always lower than that of CFC-GM while the highest frequency of CFC-DC was found in CB. The numbers of BFU-E were similar in BM and mobilized PB while CB demonstrated a 2-fold higher frequency. The PB and LP samples, which were from the same patients, were virtually identical. The frequency of CFC (DC+GM+BFU-E) from mobilized PB was marginally higher than that of CFC from BM. The highest frequency was seen with CB $CD34^+$ cells (12.8%) followed by mobilised PB $CD34^+$ cells (8.7%, 7.7% for LP) and BM $CD34^+$ cells (6.3%). The numbers of CFC-DC, GM-CFC and BFU-E were compared between BM, CB, and mobilized PB (from 13 patients). The only statistically significant differences were the higher frequencies of CFC-DC and BFU-E found in CB in comparison to BM (Fig 4.12).

Table 4.2 Total CFC Numbers per 1,000 $CD34^+$ cells (CD33 Antigen)

	CFC from $CD34^+CD33^+$ fraction	CFC from $CD34^+CD33^-$ fraction	Fold difference[†]	P value
BM (n=3)				
CFC-DC	4.3 ±2.1	6.9 ±6.8	x1	ns
CFC-GM[‡]	10.3 ±7.9	0.4 ±0.4	x36	ns
BFU-E[‡]	46.6 ±43.2	6.9 ±2.6	x7	ns
CB (n=4)				
CFC-DC	7.5 ±2.9	2.9 ±1.0	x3	ns
CFC-GM	3.7 ±2.2	1.5 ±1.4	x3	ns
BFU-E	78.9 ±28.7	98.8 ±31.2	x1	ns
PB (n=2)				
CFC-DC	7.5 ±6.4	0.7 ±0.4	x11	ns
CFC-GM	35.0 ±4.1	2.8 ±1.4	x13	ns
BFU-E	32.6 ±1.3	10.9 ±2.6	x3	ns
LP (n=2)	Same samples as 2 PB samples above			
CFC-DC	7.1 ±4.4	0.2 ±0.2	x36	ns
CFC-GM	32.1 ±5.9	6.5 ±5.4	x49	ns
BFU-E	38.3 ±16.2	30.7 ±24.5	x1	ns
LP (n=4)				
CFC-DC	5.7 ±2.1	1.4 ±1.2	x4	ns
CFC-GM	23.0 ±6.1	7.0 ±3.7	x3	ns
BFU-E	42.1 ±18.6	26.4 ±11.4	x2	ns

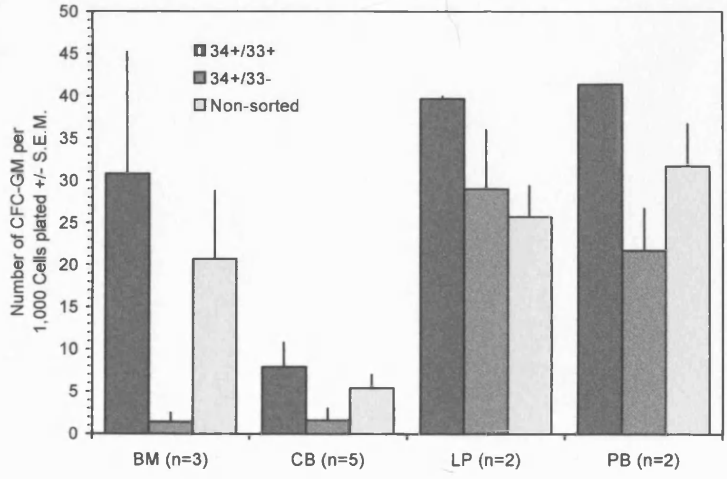
[†] Fold difference compared to single positive cells; Fold differences have been approximated to the nearest decimal point; CFC-GM data were derived from the CFC-mix assay; p values refer to comparison of total CFC numbers in $CD34^+$ cells derived from the $CD33^+$ and $CD33^-$ fractions (Mann-Whitney test); ns = not significant. [‡] For CFC-GM and BFU-E derived from BM mean CFC numbers are based on results of 2 samples.

Fig 4.9 Frequency of CFC-DC in BM, CB, LP and Mobilized PB.



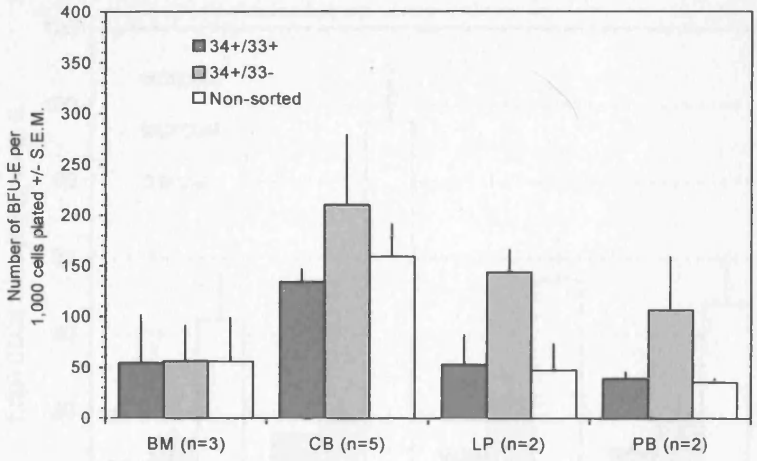
$CD34^+$ cells were sorted into $CD33^+$ and $CD33^-$ fractions in the CFC-DC assay. LP and PB samples were from the same patients.

Fig 4.10 Frequency of CFC-GM in BM, CB, LP and Mobilized PB.



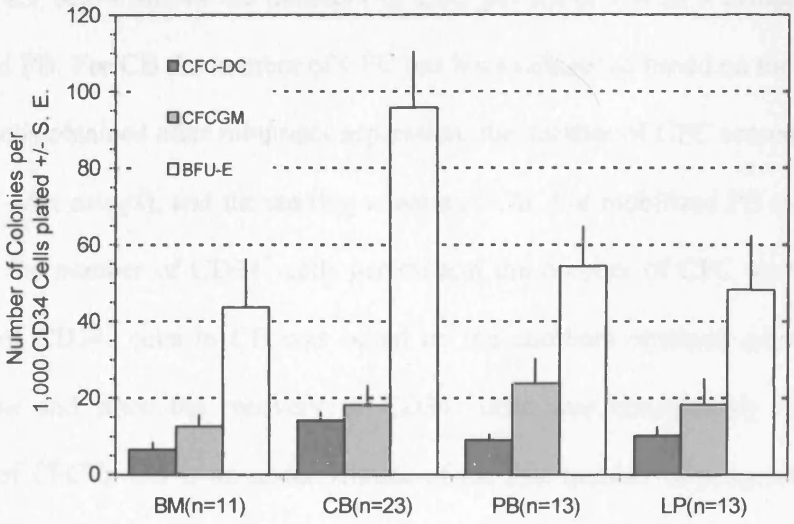
$CD34^+$ cells were sorted into $CD33^+$ and $CD33^-$ fractions in the CFC-Mix assay. LP and PB samples were from the same patients.

Fig 4.11 Frequency of BFU-E in BM, CB, LP and Mobilized PB.



$CD34^+$ cells were sorted into $CD33^+$ and $CD33^-$ fractions in the CFC-Mix assay. LP and PB samples were from the same patients.

Fig 4.12 Comparison of CFC Frequency between BM, CB and Mobilized PB



Comparison of differences in colony numbers between CB-BM, CB-Mobilized PB and BM-Mobilized PB showed that only the higher CFC-DC and BFU-E numbers in CB compared to BM were statistically significant ($p = 0.012$ and 0.021 respectively, Mann-Whitney test). The PB and LP samples were collected on the day of aphaeresis from the same patients.

4.2.5 CFC numbers per unit volume of CB and PB

Table 4.3 below shows the numbers of CFC per ml of CB in a comparison with mobilized PB. For CB the number of CFC has been calculated based on the number of $CD34^+$ cells obtained after minimacs separation, the number of CFC scored (CFC-DC and CFC-Mix assays), and the starting volume of CB. For mobilized PB the variables included the number of $CD34^+$ cells per ml and the number of CFC scored. As the number of $CD34^+$ cells in CB was based on the numbers obtained *after minimacs separation* and since the recovery of $CD34^+$ cells was considerably <100%, the number of CFC in CB is an underestimate of the true number of progenitors. Using data for PB the number of expected and actual $CD34^+$ cells obtained post immunomagnetic separation was calculated. The mean recovery of $CD34^+$ cells in PB was 38%±3 (SEM). This was calculated as follows:

$$\text{Recovery} = \frac{\text{No. of cells in "positive" final fraction x \% purity of } CD34^+ \text{ cells}}{\text{Initial no. of } CD34^+ \text{ cells in the whole blood sample}}$$

The number of $CD34^+$ cells and CFC in CB were multiplied by 2.64 to calculate the "expected" figures *assuming 100% recovery and that the initial recovery rate for CB $CD34^+$ cells post minimacs separation was identical to that of PB.* For PB this did not apply as the true number of $CD34^+$ cells per unit volume was calculated by multiplying the total WCC/ ml of PB with the % of $CD34^+$ cells in the sample.

Table 4.3 CFC and CD34$^+$ Cell Numbers /ml of CB and Mobilized PB

	CD34$^+$	CFC-DC	CFC-GM	BFU-E
	Mean ±SEM (Range)			
CB† (n=15)	**31,022 ±7,254** (8,805 – 109,970)	**366 ±99** (30 - 1,302)	**292 ±92** (7 - 1,320)	3,705 ±1,198 (0 - 14,748)
PB† (n=18)	**132,152 ±33,927** (22,140 - 485,040)	**1,481 ±611** (81 - 11,010)	**3,886 ±1,645** (0 - 27,162)	6,048 ±1,991 (119 - 35,653)
P value	**<0.001**	**0.014**	**0.013**	ns
(CB vs. PB, Mann-Whitney test)				
CB† "Expected"	81,899	966	771	9,782

† For methods of calculation see text. The PB figures have been calculated based on cell/ CFC numbers on the day of aphaeresis. Mean ±SEM (range) purity of CD34$^+$ cells was, 66% ±5 (44 - 96%) for CB and 73%±5 (41 – 96%) for PB. The differences in CFC-DC, CFC-GM and CD34 numbers between CB and PB were significant. For the "expected" CB figures there was no significant difference with PB.

4.3 Discussion

Expression of AC133 on $CD34^+$ cells

The percentage of $AC133^+$ cells among the CD34 population was lowest for BM, followed by CB and highest for mobilized PB and LP (Fig 4.1). This finding is consistent with the fact that AC133 is a marker for primitive HPC and for stem cells (Yin et al. 1997) and as such its expression would be increased in "haemopoietic tissues" rich in committed clonogenic progenitors (i.e. mobilized PB) and more primitive stem cells. The high percentage of $AC133^+$ cells among the $CD34^+$ cell population (63%, range 41-87%) is consistent with recently published data (79%, range 56-88%) by Pasino and co-workers (Pasino et al. 2000).

AC133 expression and CFC

The majority of granulo-monocytic dendritic progenitors were derived from the $AC133^+$ fraction in BM, CB and mobilized PB (including LP). Erythroid progenitors were derived primarily from the $CD34^+AC133^-$ fraction in BM although this was not statistically significant (Table 4.1). The first finding is in agreement with data from Yin and colleagues (Yin et al. 1997) and de Wynter and colleagues (de Wynter et al. 1998). The second one supports the original observation made by Yin and colleagues in 1997 (Yin et al. 1997). However this is the first report to confirm the enrichment of CFC-DC, using a clonogenic assay, within the $CD34^+AC133^+$ cell fraction in BM, CB and mobilized PB. Selection of these double positive cells that are rich in DC progenitors may therefore offer advantages, such as *in vitro* culture for generation of DC for large-scale vaccine production. In the 3 PB samples tested the ratio of $CD34^+AC133^+$ to $CD34^+AC133^-$ cells was 9:1. Moreover the frequency of CFC-DC in $CD34^+AC133^+$ cells was higher than that of CFC-DC in $CD34^+AC133^-$ cells.

Selective isolation of $CD34^+AC133^+$ cells from mobilized PB would result in a nearly 4-fold enrichment of DC progenitors from non-sorted $CD34^+$ cells; this, assuming the CFC-DC frequency in non-sorted $CD34^+$ and sorted $CD34^+AC133^+$ cells to be 4/1,000 and 13/1,000 respectively, and taking into account a 77% $CD34^+$ cell purity in the starting non-sorted population. Getting rid of "unwanted cells" ($CD34^+AC133^-$ fraction) has theoretical advantages such as minimising any *in vitro* growth inhibitory effects on CFC-DC. Additionally for large-scale DC generation, the use of cells enriched for CFC-DC may prove useful by reducing the costs of the *in vitro* culture phase of DC production.

Although in BM and CB $CD34^+AC133^-$ cells gave rise to small numbers of DC colonies, mobilized PB $AC133^-$ cells in the 3 samples tested did not. All three PB samples were processed as soon they were obtained, the viability of the sorted cells was >98% and despite plating 2 different concentrations of $CD34^+AC133^-$ cells in the CFC-DC assay (1,000 and 2,000/ ml) no DC colonies were seen. $CD34^+AC133^-$ cells -from the same samples- that were plated in the CFC-Mix assay and therefore acted as an internal control did demonstrate GM colony growth. This would suggest that the $CD34^+AC133^-$ cells from PB either contained very few or no DC progenitors (frequency <1 in 2,000 $CD34^+AC133^-$ cells) or that the growth requirements of any DC progenitors present in this cell population were markedly different from the ones used, hence failing to detect them.

CD33 Expression and CFC

Expression of CD33 by $CD34^+$ cells is already known to be found in cells committed to the myeloid lineage (Andrews et al. 1986) and therefore it is not surprising to find the $CD34^+CD33^+$ fraction to be enriched in CFC-DC, in mobilized

PB, CB and to a lesser degree BM (Table 4.2). The fold difference in the frequency of CFC-GM between $CD33^+$ and $CD33^-$ cells was much lower in mobilized blood or LP (~2-fold) than in BM (23-fold) or CB (5-fold) (Fig 4.10). When comparing the frequency of CFC in 4 LP studied for AC133 and CD33 expression, one important finding emerges; the fold difference in frequency of DC and GM progenitors was greater when comparing $AC133^+$ vs $AC133^-$ cells than $CD33^+$ vs $CD33^-$ cells (Figs 4.5a vs 4.5b, and paragraph 4.2.2 vi). This would support the use of AC133 as a marker to enrich CD34 cells for GM-DC progenitors.

Comparison of CFC frequencies between BM, CB and PB

The frequency of CFC-DC was higher in $CD34^+$ cells from mobilized PB than BM (9/1,000 vs 7/1,000 respectively). This trend was observed in another study (Ratta et al. 1998) where similar values were reported -8 and 4 CFC-DC /1,000 $CD34^+$ cells in mobilized PB and BM respectively- using the same cytokine combination. Moreover in that study the BM and PB samples were obtained from the same patients. However although in the study by Ratta and colleagues the difference was statistically significant this was not the case in this study. This could be due to sample variation or the fact that there is no significant difference when a larger number of samples are tested; 11 BM and 13 PB samples tested by the author versus 7 in each group in the study by Ratta and colleagues.

Several studies (summarized in table 4.4) have shown that CFC-DC are always lower in number than CFC-GM for a fixed number of $CD34^+$ cells plated. This finding has been confirmed by this work (Fig 4.12). Although CFC-GM growth was seen in the DC assay, the TNF-α concentrations used were such that GM progenitor growth was suppressed (chapter 3, figs 3.10-3.12). Therefore the CFC-Mix

clonogenic assay is a more precise way to measure GM progenitors *in vitro* than the DC assay. This notion is supported by what is already known about myeloid DC development, i.e. that different cytokine combinations determine the differentiation of progenitor cells. For example with GM-CSF and TNF-α $CD1a^{-}CD14^{+}$ cells develop into interstitial DC-like cells, while in the presence of M-CSF, development into macrophage-like cells occurs (introduction, fig 1.3c). Table 4.4 shows that the DC: GM progenitor ratio (1:1.9) for BM $CD34^{+}$ cells in this study, was comparable to that reported by Young and colleagues (1:1.5) using the CFC-DC assay alone (Young et al. 1995).

The highest frequency of CFC-DC was found in CB $CD34^{+}$ cells. The higher number of DC plus GM progenitors per 1,000 $CD34^{+}$ cells in CB compared to BM (32 vs 19) suggests that there is a higher proportion of multipotent -bipotent (GM) or tripotent (GMDC)- progenitors in CB. This would be entirely in agreement with previous work (Bender et al. 1994; Mayani et al. 1998) that has shown that compared to BM CB contains a higher proportion of CFC (Mayani et al. 1998; Mayani and Lansdorp 1998) and primitive–cell colonies like BFU-E (Hows et al. 1992). The levels of erythroid progenitors were 2-fold higher in CB than in BM or mobilized PB. Previous studies (Hows et al. 1992; Mayani et al. 1998; Steen et al. 1994) have also found higher levels of BFU-E -the more primitive type of erythroid colony- in CB.

Table 4.4 Comparison of the CFC-DC: GM ratio obtained in this study with previous studies.

	BM	CB	PB
Main author of study	Ratio of CFC-DC: GM (% Purity for $CD34^+$ cells in sample)		
(i) Reid (Reid et al. 1992)	1:3.6[†] (n=6)	N/D	N/D
FACS sorted $CD34^+$ cells	(66% ±10)		
(ii) Ratta (Ratta et al. 1998)	1:3.3 (n=7)	N/D	1:4.9 (n=7)
Immunomagnetically separated	96%		96%
$CD34^+$ cells	(SEM, not		(SEM, not
	given)		given)
(iii) Young (Young et al. 1995)	1:1.5 (n=7)	N/D	N/D
Immunomagnetically separated	(purity not		
$CD34^+$ cells	given)		
(iv) Author	**1:1.9 (n=11)**	**1:2.6 (n=23)**	**1:2.7 (n=13)**
Immunomagnetically separated	(84% ±2[‡])	(67% ±10[§])	(77% ±5)
$CD34^+$ cells			

Results are expressed as the ratio of CFC-DC to CFC-GM. CFC-GM values for author's data were derived from the CFC-Mix assay (n = number of experiments, mean purity of $CD34^+$ cells ±SEM); [†] purity not determined (N/D) for 2 samples; [‡] purity N/D for 1 sample; [§] purity N/D for 3 samples.

CFC numbers per unit volume of blood

Table 4.3 shows that per unit volume PB is superior to CB in CFC content. This is because of the significantly higher numbers of $CD34^+$ cells in mobilized PB. The mean CFC-GM and BFU-E numbers (PB) on the day of aphaeresis were comparable to previously published data derived by plating MNC in patients undergoing HPC mobilization with chemotherapy and G-CSF (Pettengell et al. 1993a). As regards CB, the "expected" figure for BFU-E (9,776/ ml, table 4.3) was similar to the reported figure of 8,000 in the literature (Mayani and Lansdorp 1998). The reported figure quoted for CFC-GM (13,000-24,000/ ml) in the same study was considerably higher. Likely reasons for this are:

1. Differences in culture conditions, for example addition of SCF is known to increase detection of myeloid progenitor cells (Broxmeyer et al. 1992).
2. Because of logistical reasons cord blood samples were 24 - 48 hours old and therefore some CFC-GM would be non-viable.
3. Large losses of HPC occur as a result of cell separation (Gluckman et al. 1993).

Published data for aphaeresis products have shown the reduction in viability of CFC-GM to be 18% and 31% at 24 and 48 hours respectively (Pettengell et al. 1994). These reductions may be even greater for CB CFC-GM in the absence of G-CSF, a cytokine known for promoting the survival of HPC (Nicola 1990) and which would be present in the serum contained in aphaeresis products. To the author's best knowledge this is the first study to have assayed extensively CFC-DC in CB and there are no published data of DC progenitor numbers/ unit volume of CB for comparison. The number of CB CFC-GM above may be an underestimate of the true number while that for CFC-DC is thought to reflect the true value more closely as recombinant

growth factors (including SCF) were employed. It is of interest however that 2 other studies quoted CB CFC numbers that are more comparable to the author's data than to those reported by Mayani and Lansdorp (Mayani and Lansdorp 1998):

1. Broxmeyer data: CFC-GM 2,209/ ml (n=65, GM-CSF stimulated cultures in agar) (Broxmeyer 1998).
2. Gluckman data: CFC-GM 1,850/ ml, BFU-E 1,050/ ml (n=75, culture conditions not reported) (Gluckman et al. 1993).

These figures are only 2-3 fold higher than the author's "expected" CB data and a lot lower than those reported by Mayani (Mayani and Lansdorp 1998). Differences in culture conditions as well as sample variation are possible reasons. It is also possible that if a larger number of CB samples were tested in this study the GM progenitor content might have been closer to Broxmeyer's and Gluckman's data. The number of $CD34^+$ cells/ ml CB (31,000 ±SEM 7,000, n=15) is comparable to the figure recently reported (56,000 ±SEM 39,000, n=6) in a study (Campagnoli et al. 2000) where $CD34^+$ cells were enumerated with the same method (ISHAGE guidelines).

Best source of CFC-DC and implications

PB is therefore the best and most easily accessible source of DC progenitors. This has important implications in the design of immunotherapy protocols for treating cancer. It supports the generation of DC from $CD34^+$ HPC using conventional chemotherapy and r-HGFs in patients enrolled in high-dose chemotherapy trials. This approach however could also be applied to most patients with malignant disease who require chemotherapy, in whom DC progenitors could be mobilized by administration of G-CSF and obtained by addition of aphaeresis to their treatment. Mobilization regimens therefore that are most effective for $CD34^+$ or $CD34^+AC133^+$ cells might be

expected to be ideal for mobilizing large numbers of CFC-DC. Collection of CFC-DC when release into PB is at its peak is therefore crucial. Hence time course studies were conducted to identify this and to determine the number of CFC-DC/ kg body weight in LP (chapter 5).

4.4 Conclusions

The following observations were made:

- The percentage of $AC133^+$ cells within the CD34 population varied. For BM and PB at steady state this was 35-40%, for CB 63% and for mobilized PB/LP 78%.
- **CFC-DC** and CFC-GM were mainly $CD34^+AC133^+$ in the BM, CB and mobilized PB. CD33 was not uniquely expressed by myeloid CFC-DC, and $CD34^+CD33^+$ cells only had an ~2-fold higher frequency of CFC-DC than $CD34^+CD33^-$cells.
- BFU-E were primarily $CD34^+AC133^-$ cells. The predominance of $CD34^+AC133^+$ cells however, meant that in mobilized PB there were more BFU-E in the double positive fraction as a whole, while in CB they were equally distributed between the $AC133^+$ and $AC133^-$ cells. In BM the majority of BFU-E was within the $AC133^-$ cell population.
- CB $CD34^+$ cells showed the highest frequency for CFC-DC (1.4%) with mobilized PB being second (0.9%) and BM third (0.7%). Mobilized PB $CD34^+$ cells had the highest frequency of CFC-GM (2.4%) with CB being second (1.8%) and BM third (1.3%).
- The richest and most easily accessible source of DC progenitors was PB $CD34^+$ cells mobilized with myelosuppressive chemotherapy and G-CSF. The high numbers of CFC-DC in PB were mainly due to the high numbers of $CD34^+$ cells.

- This data allows us to calculate the number of CFC-DC that would need to be collected from mobilized PB. For example 400ml, which could be obtained by venesection, would contain on average $0.6x10^6$ CFC-DC. With a 40-fold expansion this would yield $24x10^6$ DC. This figure is very much near the median $30x10^6$ DC used for immunization in the first published and successful clinical trial that used autologous antigen-pulsed DC (Hsu et al. 1996).

Chapter 5

The Mobilization Kinetics of Myeloid DC Progenitors in Patients with Solid Tumours Receiving Chemotherapy and G-CSF

5.1 Introduction

Dendritic cell based immunotherapy is an exciting and relatively novel therapeutic modality that is currently under evaluation in phase I and II clinical trials (reviewed in (Brossart et al. 2001; Luykx-de Bakker et al. 1999; Morse and Lyerly 1998; Sprinzl et al. 2001)) with the potential for use in phase III studies in the near future. One of the difficulties in this field is the availability of sufficient numbers of DC in the clinical setting because of their low numbers in PB (Fearnley et al. 1999; Reid 1997). This has been overcome to some degree by the *ex vivo* expansion of $CD34^+$ HPC using one of several cytokine combinations, usually GM-CSF and TNF-α ±SCF ±FLT-3L (Caux et al. 1992; Caux et al. 1996a; Siena et al. 1995; Szabolcs et al. 1996; Szabolcs et al. 1995) or by the differentiation of Monocytes into Mo-DC with GM-CSF and IL-4 (Chapuis et al. 1997; Romani et al. 1994; Romani et al. 1996; Sallusto et al. 1995; Sallusto and Lanzavecchia 1994; Zhou and Tedder 1996). The increasing use of chemotherapy and CSFs (e.g. G-CSF) to mobilize large numbers of these HPC is an essential part of several cancer treatment protocols that have been developed over the last decade (Hohaus et al. 1998). As early as 1995 it was recognised that the use of mobilized $CD34^+$ PBPC as part of an intensified chemotherapy regimen could be combined with the *ex vivo* generation of DC (Siena et al. 1995). These would be pulsed with clinically relevant antigens and infused into patients to stimulate

cytotoxic T-cell responses (Steinman 1996) thus conferring a dual therapeutic benefit alongside the $CD34^+$ cells used for haematopoietic reconstitution.

Progenitors of DC like other haemopoietic progenitors lack a single and specific marker so the only means of assaying them, in order to establish the timing of their highest numbers in PB, is using a clonogenic assay. To date there have been no studies examining the kinetics of mobilization of clonogenic DC progenitors. One study has assayed DC progenitors in leukaphaeresis products (Siena et al. 1995). This study used a limiting dilution analysis and a mobilization protocol that included IL-3 with G-CSF. IL-3 is an infrequently used cytokine because of unpleasant side effects and insufficient evidence on whether it is advantageous over G-CSF alone (To et al. 1997). In this study the mobilization kinetics of DC progenitors were investigated in cancer patients undergoing HPC mobilization, with chemotherapy and G-CSF, as part of their anti-tumour therapy.

Two recent observations (de Wynter et al. 1998; Decatris et al. 1999) have clearly shown that most clonogenic myeloid DC precursors are not only $CD34^+$ but also express the stem cell antigen, AC133 (Yin et al. 1997). Therefore time course studies examined the kinetics of release of $AC133^+$ cells into PB in comparison to those of $CD34^+$ cells. Patients with solid tumours were chosen because of easy access, and greater likelihood to mobilize well than patients with haematological malignancies. All patients underwent HPC mobilization with conventional dose chemotherapy and G-CSF as part of their treatment within ethically approved clinical trials.

5.2 Patients, materials and methods

5.2.1 Patients and blood sampling

The numbers of clonogenic progenitors of dendritic (CFC-DC), granulocyte-macrophage (CFC-GM) and erythroid cells (BFU-E) in blood was determined at steady state and after mobilization. Patients with solid tumours enrolled in phase III trials of high dose chemotherapy were studied. Of the 21 patients studied in total, 13 had metastatic nonseminomatous germ cell tumour (GCT), 7 had breast cancer and 1 patient had non-Hodgkin's lymphoma (NHL). All patients had histologically confirmed tumours. The GCT patients were of intermediate or high risk as defined by the International Consensus classification (The International Germ Cell Cancer Collaborative Group 1997) and were undergoing progenitor cell mobilization with a view to high dose chemotherapy (HDCT)/ PBPCT. The breast cancer patients had HPC mobilization as part of their treatment for metastatic disease or adjuvant therapy for poor prognosis disease (histological involvement of 4 or more axillary lymph nodes). All patients had given written informed consent prior to entry into the HDCT studies, which had the approval of the local ethics committee. Blood samples were taken, with the patients' informed consent, simultaneously with clinical samples to avoid additional venepunctures as far as possible. $CD34^+$ and $AC133^+$ cells were enumerated. Aphaeresis products (LP) were similarly analysed. PB, BM and LP samples were obtained as described in chapter 2 (section 2.1.2).

5.2.2 Progenitor cell mobilization

For HPC mobilization patients received conventional dose cytotoxic chemotherapy (Table 5.1). The first day of chemotherapy was designated day 1. The GCT group received Cisplatin-based chemotherapy (BEP, Bleomycin/ Etoposide/ Cisplatin or

VIP, Etoposide/ Ifosfamide/ Cisplatin) followed by rh-G-CSF (Lenograstim (Chugai®), Japan) 5μg/ kg /d subcutaneously (s/c). Patients with metastatic breast cancer received Adriamycin/ Taxotere (AT) and those with non-metastatic disease, 5-Fluorouracil/ Epirubicin/ Cyclophosphamide (FEC) followed by rh-G-CSF 263 μg/ d s/c as per protocol; aphaeresis was performed when the leukocyte count was rising from nadir and was above 3 x 10^9/ l. Patients with GCT and non-metastatic breast cancer had aphaeresis usually after their first cycle of chemotherapy or if this was not possible for logistical reasons after their second cycle. The 2 patients with disseminated breast cancer were harvested after cycle 2 in accordance with the HDCT study protocol (European Breast Dose Intensity Study-1, EBDIS-1). MNC were isolated as described in section 2.1.2b and plated in CFC-DC and CFC-Mix clonogenic assays as described in section 2.1.3. DC identity was confirmed using Giemsa staining and immunocytochemistry for known DC markers CD83, CD86 and HLA-DP-DR as described in section 2.2.3.

5.3 Results

5.3.1 Patient characteristics

A total of 21 patients were studied with a median age of 35 (Table 5.1). The median number of aphaereses was 1 (range 1-2). One GCT patient with a body weight of 120kg required harvesting over 2 consecutive days in order to collect sufficient cells. Two patients with metastatic breast cancer had 2 collections in accordance with the protocol of the HDCT study in which they were enrolled. One breast cancer patient had a repeat aphaeresis because of poor mobilization after the first chemotherapy cycle while another breast cancer patient had harvesting over 2 consecutive days because the first day's collection was poor.

Table 5.1 Characteristics of all Patients Studied.

Disease Group / UPN †	Age	Stage (IGCCCG ‡ Prognostic Group)	Previous Chemotherapy§	Mobilization Chemotherapy‖	Harvest (Day)
GCT					
01	18	m (inter)	None	BEP #1	1 (d14)
02	25	m (inter)	None	BEP #1	1 (d14)
03	34	m (poor)	None	BEP #1	1 (d18)
04	40	m (inter)	None	BEP #1	1 (d13)
05	35	m (inter)	None	BEP #1	1 (d14)
06	21	m (inter)	BEPx1	BEP #2	1 (d16)
07	31	m (poor)	BEPx1	BEP #2	1 (d14)
08	35	m (inter)	BEPx1	BEP #2	1 (d15)
09	37	m (poor)	BEPx1	BEP #2	1 (d14)
10	33	m (good/ rel)	BEPx3	VIP #2	1 (d16)
11	42	m (inter/ rel)	BEPx3	VIP #2	1 (d14)
12	32	m (good/ rel)	BEPx3	VIP #2	2 (d12+13)
13	32	m (poor)	None	N/A	N/D
Breast cancer					
14	21	nm (poor)	None	FEC #1	1 (d11)
15	49	nm (poor)	None	FEC #1	2 (d11+12)
16	35	nm (poor)	None	FEC #1	1 (d11)
17	48	nm (poor)	FECx1	FEC #2	2 (d11)
18	53	nm (poor)	FECx1	FEC #2	1 (d11)
19	42	m	CMFx6	AT #2	2 (d9+10)
20	49	m	CMFx6	AT #2	2 (d9+10)
NHL					
21	35	III	VAPEC-B (11 weeks)	CHOP #3	1 (d11)

N=21; median age = 35; **patients who had kinetic studies in bold.**

† Unique patient number; ‡ IGCCCG = International Germ Cell Cancer Collaborative Group, m = metastatic, nm = non-metastatic, inter = intermediate, rel = relapsed; § excludes ongoing "recent" chemotherapy; ‖ BEP = bleomycin 30mg d2, 9, 16, etoposide 100mg/ m^2 d1-5, cisplatin 20mg/ m^2 d1-5; VIP = etoposide 75mg/ m^2/ d, ifosfamide 1.2gm/ m^2/ d, cisplatin 20mg/ m^2/ d, all d1-5; FEC = 5-fluorouracil 600mg/ m^2, epirubicin 50mg/ m^2, cyclophosphamide 600mg/ m^2, all on d1; AT = adriamycin 50mg/ m^2, taxotere 75mg/ m^2, both on d1; CHOP = cyclophosphamide 750mg/ m^2, adriamycin 50mg/ m^2, vincristine 1.4mg/ m^2, all on d1, prednisolone 100mg/ d (d1-5). N/A= not applicable; N/D= not done as patient died before aphaeresis.

5.3.2 Progenitor numbers before chemotherapy. Baseline values

Table 5.2 shows the pre-treatment data for a group of 12 patients. Only 2 of these had received prior chemotherapy but this was more than one year earlier. The median (range) and mean numbers of CFC-DC, in PB at steady state were, 17 (0-68) and 24/ ml respectively. The corresponding figures for GM progenitors were 40 (0-170) and 67/ ml of PB. These numbers (median) translate to 1 CFC-DC and 2.4 CFC-GM per 10^5 MNC from PB. The median number of steady-state MNC, $CD34^+$ cells, CFC-GM and BFU-E in PB was comparable to that of 2 previous studies reported for patients with NHL (Pettengell et al. 1993a) and ovarian cancer (Weaver et al. 1996) and agreed with data in the literature (McCarthy and Goldman 1984; Ogawa et al. 1977; Socinski et al. 1988). The median number of $AC133^+$ cells at steady state was only 33% of the corresponding figure for $CD34^+$ cells.

5.3.3 Kinetics of mobilization after chemotherapy and G-CSF

(i) Kinetics of CFC release

The results of detailed time course studies, which were undertaken in ten patients, (UPN 2, 3, 7, 9, 10, 12, 15, 18-20) are shown in Figs 5.1 and 5.2. Fig 5.1a-c shows the kinetics of mobilization of CFC-DC, CFC-GM and BFU-E together with changes in the WCC (Fig 5.1d) for 4 breast cancer patients. A clear decline, which coincided with the WCC nadir, was seen in CFC-DC and CFC-GM numbers (Figs 5.1a-b respectively), the week after completion of chemotherapy. In the GCT group a decline in progenitor numbers was seen following 5 days of chemotherapy (Figs 5.2a-c respectively). For one GCT patient (UPN 12) it is likely that the "progenitor nadir" was missed because blood sampling was not possible between days 5-10. In both groups the release of circulating progenitors into PB preceded the rapid rise in the WCC by 24-48 hours.

Table 5.2 Steady-state Values of $CD34^+$, $AC133^+$ Cells and Clonogenic Progenitors in 12 Patients with GCT (n=8) and Breast Cancer (n=4).

Cell Numbers / ml Blood (n=12)	Median	Mean	SEM	Range
Age	35	36		18-53
WCC $x10^6$	8.2	9.9	±1.1	6.3-17.1
MNC $x10^6$	1.7	1.9	±0.2	1.1-3.3
CD34 $x10^3$	2.2	2.5	±0.4	0.8-6.0
AC133 $x10^3$	0.7	1.1	±0.3	0-3.8
BFU-E	288	374	±105	39-1,325
CFC-GM	40	67	±20	0-174
CFC-DC	17	24	±8	0-68

For two breast cancer patients (UPN 19, 20) who were mobilized with AT the numbers of CFC-DC and CFC-GM began rising (Fig 5.2a-b respectively), by more than ten fold as early as 1-week post chemotherapy. This is consistent with an earlier and shorter period of myelosuppression that is experienced after chemotherapy with taxanes in general and which is followed by a rapid recovery of the WCC. Coexisting neutropenic sepsis, as was the case in one patient with a GCT, delayed progenitor cell release until the infection resolved.

(ii) Timing of maximal CFC-DC release

In all 10 patients the day of peak circulating CFC-DC (>98% highest numbers reached) was within 24 hours of the day of aphaeresis and in 8/10 patients on the same day. The highest CFC-DC numbers were on day 15 (median, range 12-18) for the GCT patients and on day 11 (median, range 9-11) for the breast cancer patients. The difference was probably because of the longer duration of chemotherapy for the former group of patients. When this is taken into account it becomes clear that maximal numbers of DC progenitors can be collected 10 days (median, range 7-13) after completion of chemotherapy. For these patients the median peak progenitor numbers were 330/ ml for CFC-DC, 984/ ml for CFC-GM and 2,241/ ml for BFU-E. The median fold increases over baseline were 19-fold, 12-fold and 6-fold respectively above pre-treatment values. The median WCC on the day of peak circulating DC was $6.5x10^6$/ ml (range, 3.0-37.2) and the MNC 2.8 $x10^6$/ ml (range, 0.7-10.8) for the GCT patients. For the breast cancer group the respective figures were 17.3 $x10^6$/ ml (range, 4.3-34.7) and 2.0 $x10^6$/ ml (range, 0.8-4.9).

(iii) Kinetics of $CD34^+$ and $AC133^+$ cell mobilization

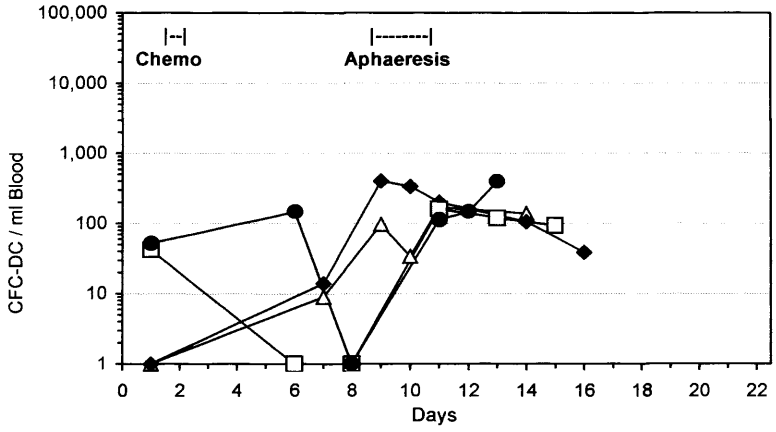
The kinetics of $CD34^+$ and $AC133^+$ cell mobilization are illustrated in Figs 5.1e-f and 5.2e-f for the breast cancer and GCT patient groups respectively. The mobilization patterns of $CD34^+$ and $AC133^+$ cells were very similar. One important difference observed however was that $AC133^+$ cells could not be detected by flow cytometry in 1 GCT and 3 breast cancer patients at steady state. In three other patients (one with breast cancer and two with GCT) the number of $AC133^+$ cells reached zero following chemotherapy. In one of these cases (UPN 7), the numbers of $CD34^+$ and $AC133^+$ cells declined from 207,870/ ml and 169,000/ ml pre-cycle 2 to 2,010 / ml and 0 /ml respectively after 5 days of chemotherapy. $CD34^+$ cells were always detected in 69 measurements, which were made for the 10 patients who had kinetic studies.

(iv) CFC Content of 20 aphaeresis products

The CFC numbers of 20 LP from 15 patients were studied (Table 5.3). The patient group included 9 patients with metastatic GCT and 6 with breast cancer (of whom 2 metastatic). CFC-DC numbers were approximately one fourth of the CFC-GM numbers although there was considerable interpatient variation.

Fig 5.1a-c Kinetics of CFC in 4 Breast Cancer Patients.

A



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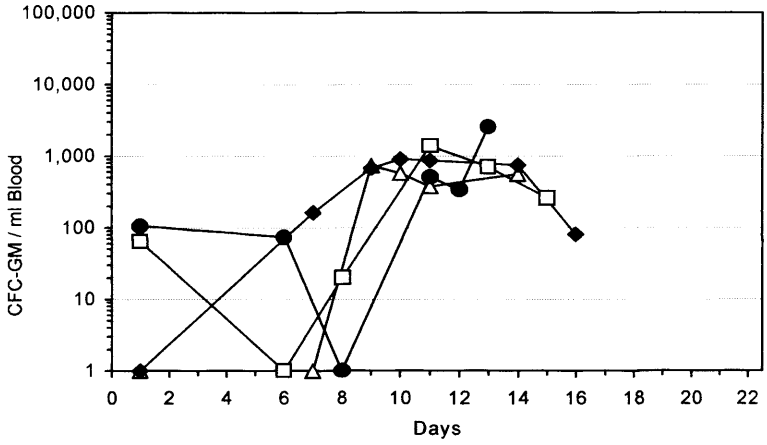

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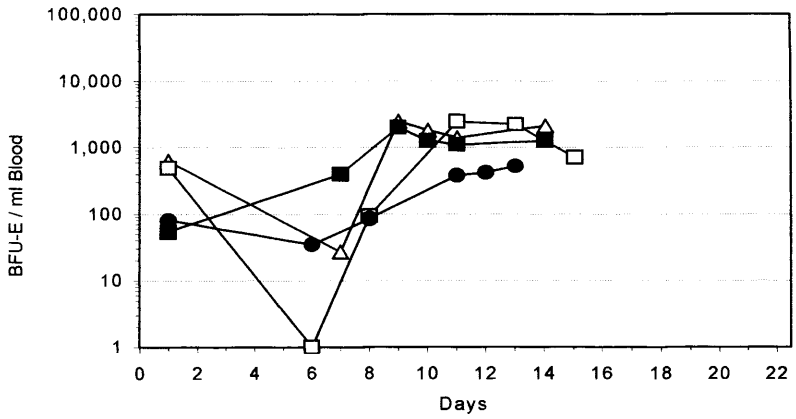
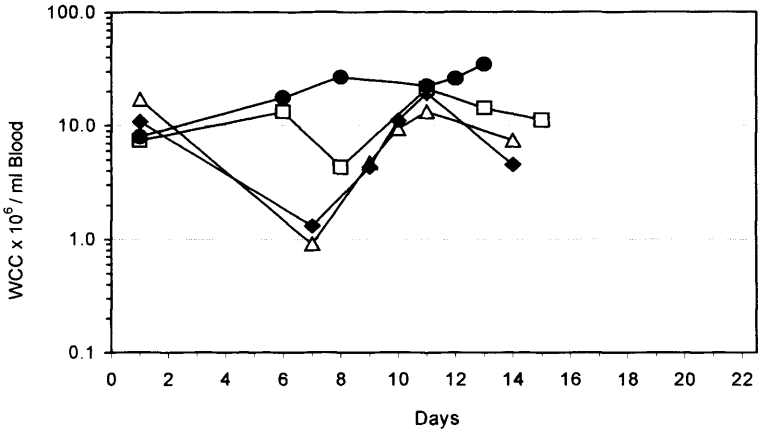


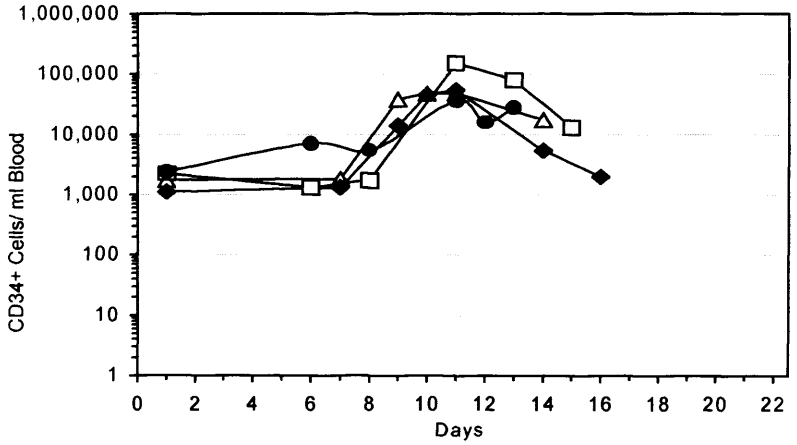
Values below the level of detection of the assay (i.e. no events observed) were assigned 1 for the purposes of presentation.

Fig 5.1d-f Kinetics of Leucocyte, $CD34^+$ and $AC133^+$ cells in 4 Breast Cancer Patients.

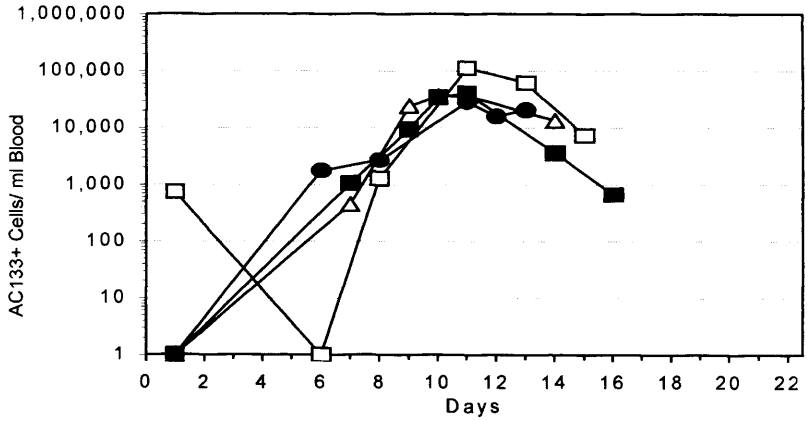
D



E



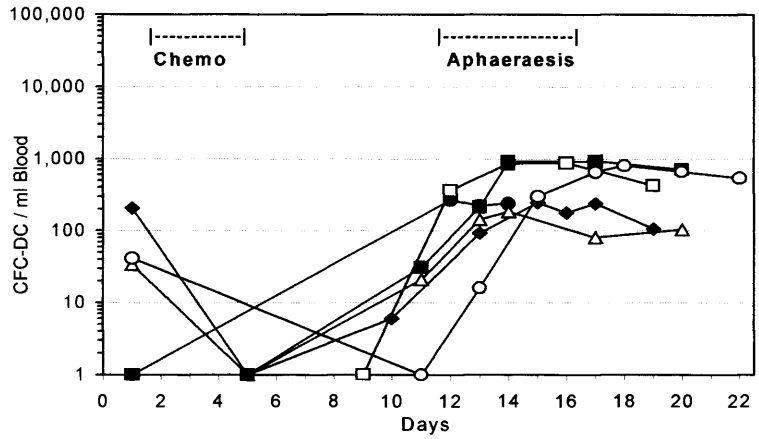
F



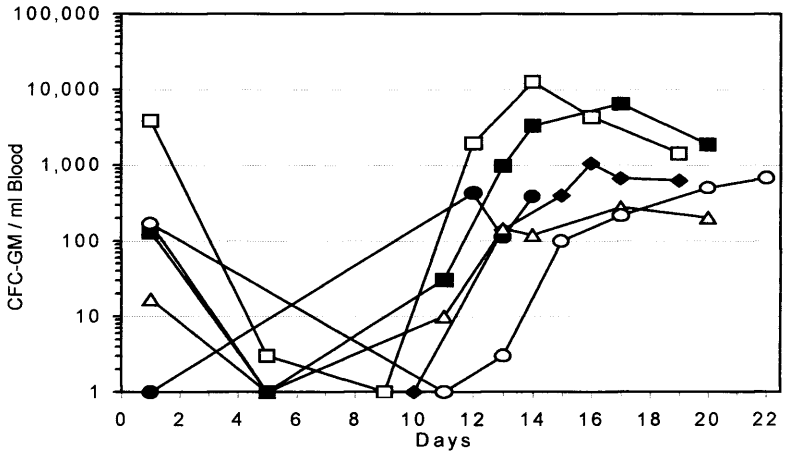
Values below the level of detection of the assay (i.e. no events observed) were assigned 1 for the purposes of presentation.

Fig 5.2a-c Kinetics of CFC in 6 Germ Cell Tumour patients.

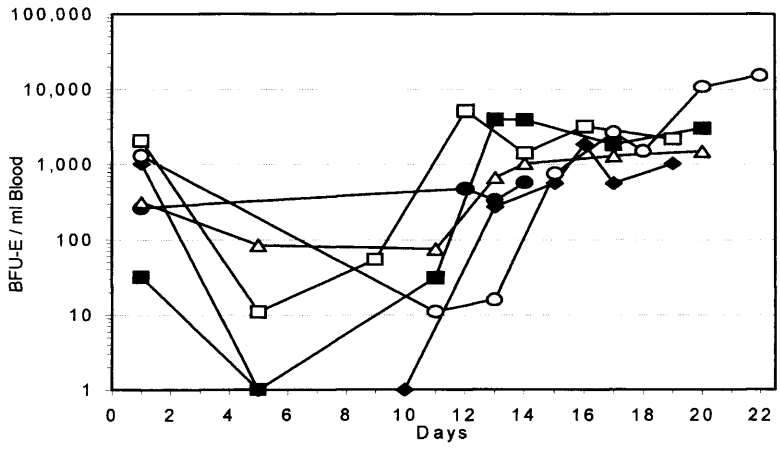
A



B



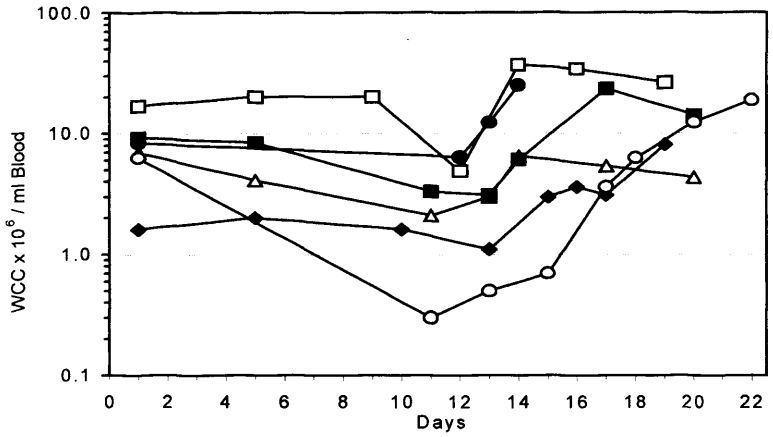
C



Values below the level of detection of the assay (i.e. no events observed) were assigned 1 for the purposes of presentation.

Fig 5.2d-f Kinetics of Leucocyte, $CD34^+$ and $AC133^+$ Cells in 6 GCT Patients.

D



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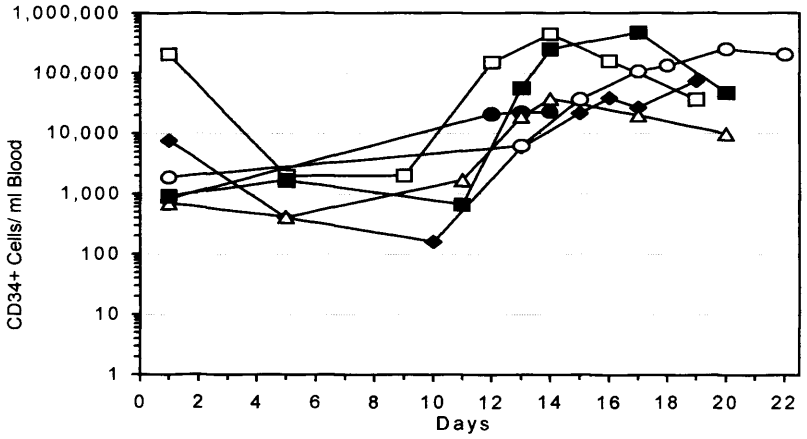
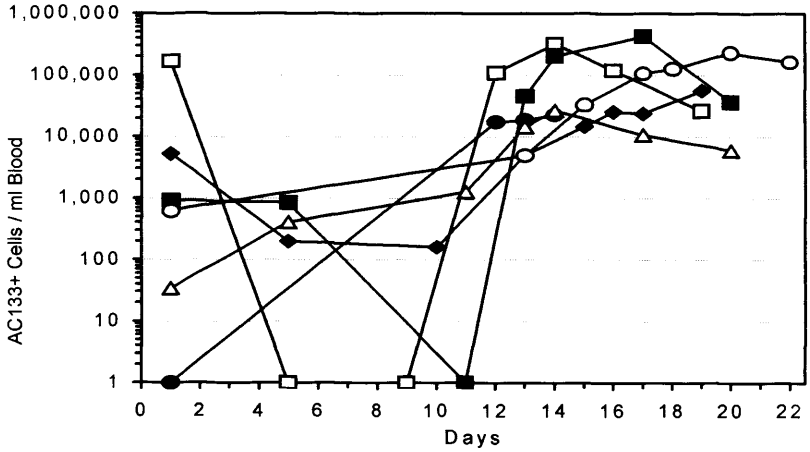


F



Values below the level of detection of the assay (i.e. no events observed) were assigned 1 for the purposes of presentation.

Table 5.3 Content of 20 Aphaeresis Products from 15 Patients[†]

Cells/ Kg body weight (n=20)	Median	Mean	SEM	Range
MNC $x10^8$	3.4	3.8	±0.4	0.8-6.1
CD34 $x10^6$	5.2	7.4	±1.5	0.4-19.9
AC133 $x10^{6‡}$	4.1	5.9	±1.3	0.3-16.8
BFU-E $x10^4$	26.2	41.3	±13.2	2.7-278.0
CFC-GM $x10^4$	14.0	25.3	±5.0	2.7-74.4
CFC-DC $x10^4$	3.8	4.7	±0.7	0.5-12.5
Frequency of CFC-DC in $CD34^+$ cells	1/111	1/84	95% confidence interval for Mean = 1/60-1/142	

[†]5 patients had 2 harvests; [‡]AC133 results based on 18 aphaeresis products from 14 patients

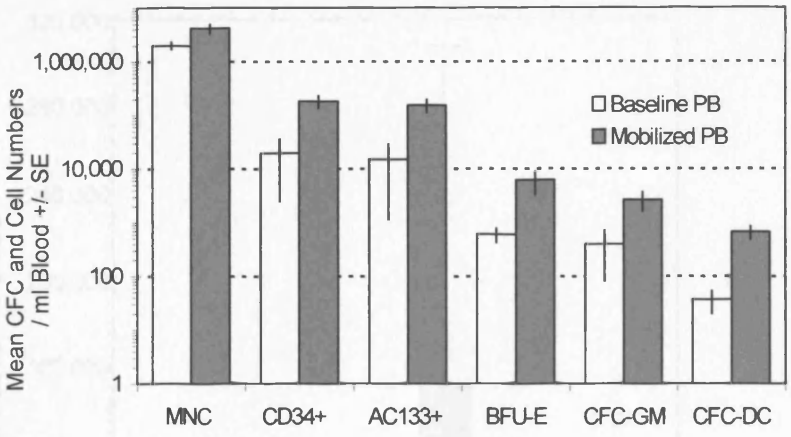
5.3.4 Progenitor cell numbers at steady state and peak mobilization in PB

Circulating DC, GM and erythroid progenitors were assayed at baseline and after mobilization in 8 patients with GCT and 4 with breast cancer (Fig 5.3a). When comparing the peak number (mean) of MNC, $CD34^+$ and $AC133^+$ cells mobilized with baseline levels the fold increases were 2.1, 9.4 and 10.2 respectively. The corresponding fold increases for BFU-E, CFC-GM and CFC-DC were 10, 6.7 and 18 respectively. Of these 12 patients 3 had baseline blood taken before cycle 2 and steady state haemopoiesis in 2 of them (UPN 7, 10) had not been re-established. When these two patients were excluded from the above analysis the fold increases from pre-treatment levels (mean values) were considerably higher, 88x for $CD34^+$ cells, 207x for $AC133^+$ cells, 17x for BFU-E, 25x for CFC-GM and 29x for CFC-DC (Fig 5.3b).

5.3.5 Progenitor cell numbers before the 1st and 2nd chemotherapy cycles

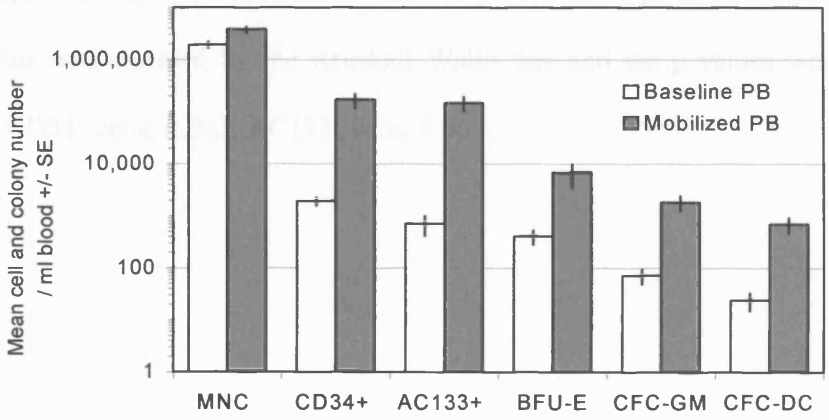
In order to establish the effect of one cycle of chemotherapy, progenitors were assayed before the first and second cycles of chemotherapy in a small group of 6 patients. The treatment gap was 21 days (16 days for 2 patients). Figs 5.4a-b show there was only a small increase in the number of cells and CFC from cycle 2 onwards whereas the difference from steady state levels prior to cycle 1 was considerably bigger. Comparison of median numbers of progenitors after mobilization, with "pre-cycle 2" levels showed a 2-fold increase for BFU-E, 7-fold for CFC-GM and 2-fold for CFC-DC. When the comparison is made with "pre-cycle 1" steady state the respective increases were 7, 26 and 6-fold. A similar pattern was seen with the numbers of CD34 and AC133 positive cells which increased 9 and 13 times respectively following mobilization after cycle 2; when compared with "pre-cycle 1" baseline, the corresponding increases were higher, 22 and 94 times respectively.

Fig 5.3a MNC and Progenitor Cell Numbers in PB at "Steady State" and Peak Mobilization in 12 Patients.



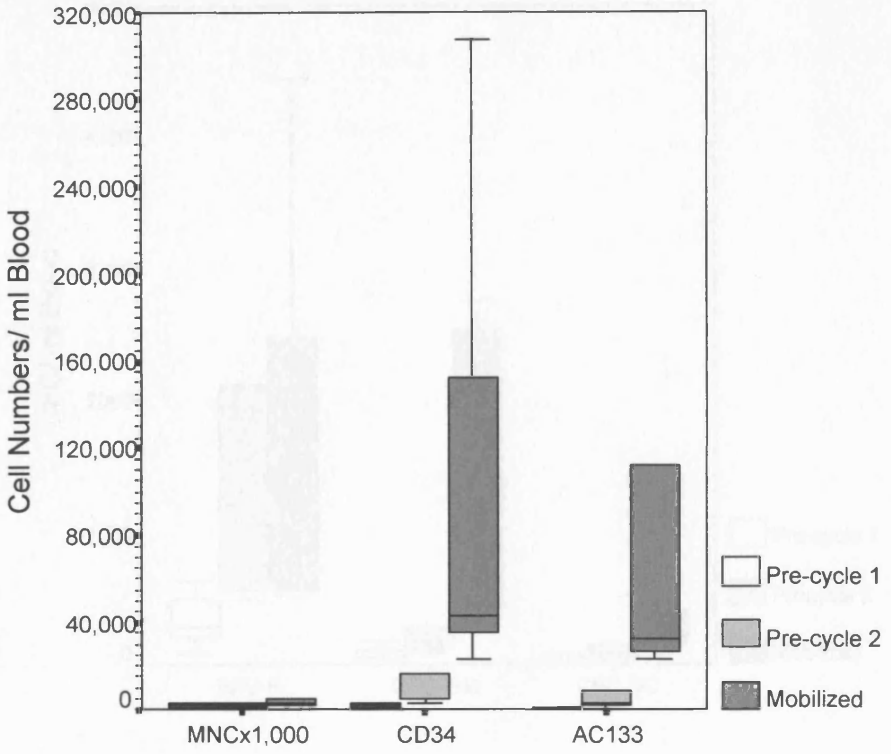
The CFC data after mobilization for 2 patients included were derived after plating $2x10^3$ $CD34^+$ cells in the CFC-DC and CFC-Mix assays. The differences between baseline and mobilized state were significant (p=0.002, Wilcoxon Matched-pairs test).

Fig 5.3b MNC and Progenitor Cell Numbers in PB at Steady State and Peak Mobilization in 10 of the above 12 Patients[†].



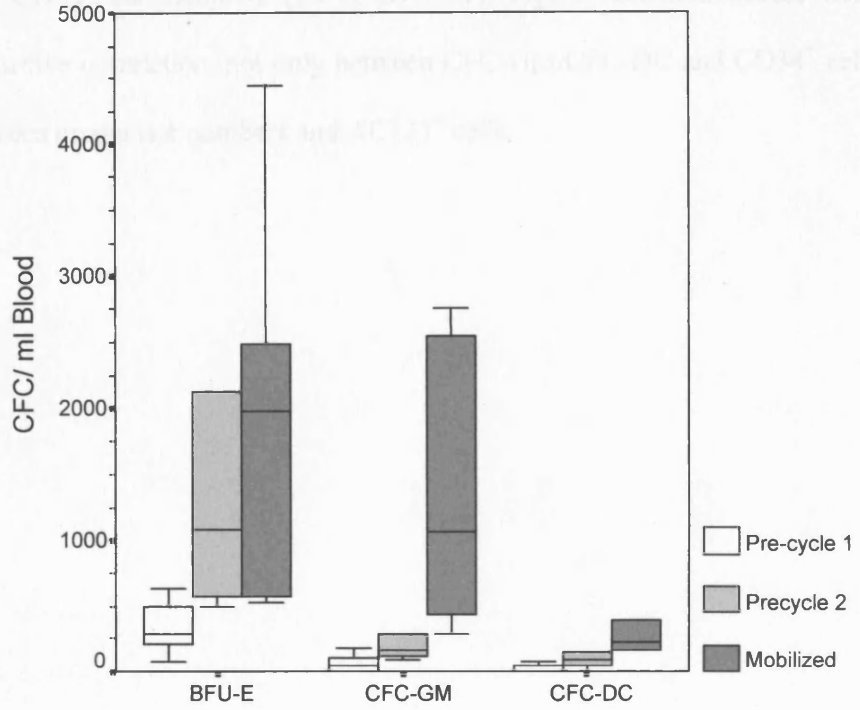
[†] Excluding the 2 cases where steady state haemopoiesis had not been re-established. The differences between baseline and mobilized state were significant (p=0.005, Wilcoxon Matched-pairs test).

Fig 5.4a Cell Numbers (per ml of PB) before Chemotherapy Cycles 1 and 2 and after Mobilization (n=6).



The horizontal bar denotes the median value and the box plot denotes the 25th and 75th percentiles and the error bars the 5th and 95th percentiles. The significance between the medians was assessed by the Kruskall Wallis test and the p values were: MNC, > 0.05; $CD34^+$ cells, 0.002; $AC133^+$ cells, 0.009.

Fig 5.4b CFC Numbers (per ml of PB) before Chemotherapy Cycles 1 and 2 and after Mobilization (n=6).

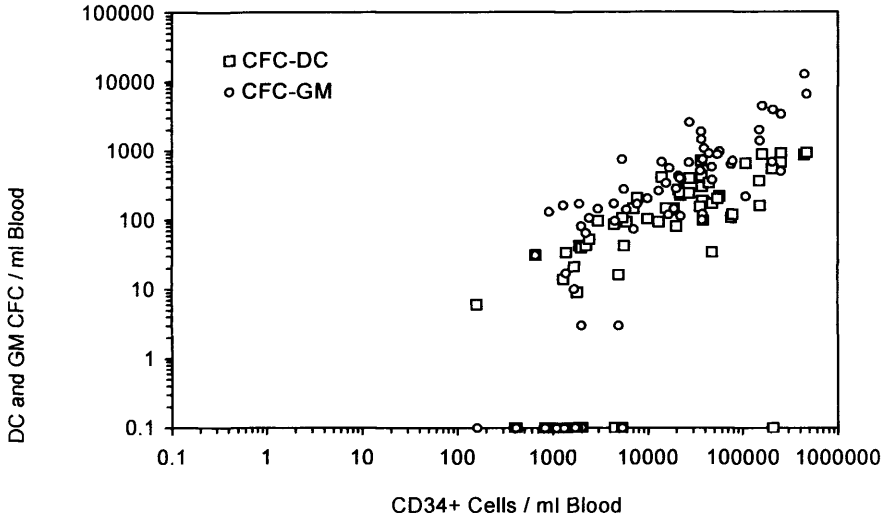


The horizontal bar denotes the median value, the box plot denotes the 25th and 75th percentiles and the error bars the 5th and 95th percentiles. The significance between the medians was assessed by the Kruskall Wallis test and the p values were: BFU-E, 0.135; CFC-GM, 0.009; CFC-DC, 0.002.

5.3.6 Correlation of CFC-DC/ CFC-GM with PB $CD34^+$ and $AC133^+$ numbers

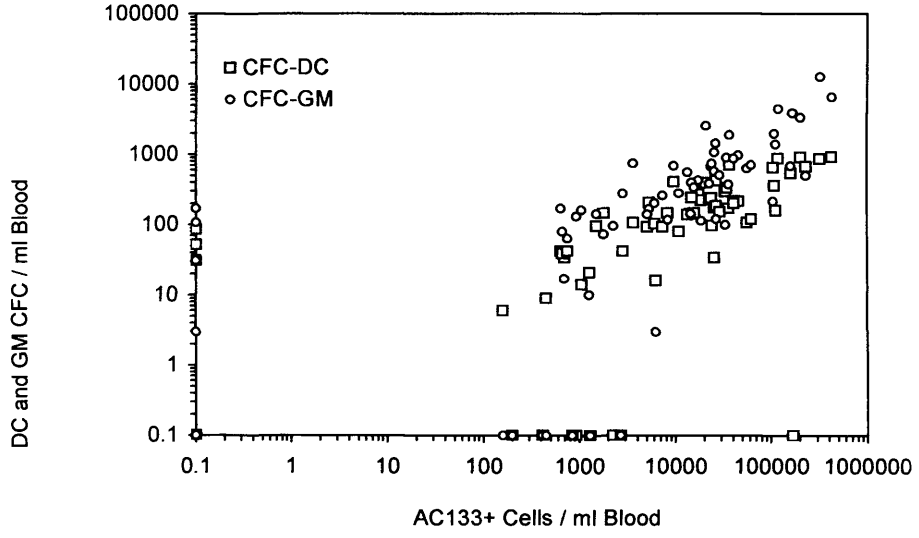
Numerous studies have confirmed a highly significant correlation between CFC-GM and $CD34^+$ cell numbers (To et al. 1997). Figs 5.5a-b demonstrate clearly a strong positive correlation, not only between CFC-GM/CFC-DC and $CD34^+$ cells but also between progenitor numbers and $AC133^+$ cells.

Fig 5.5a-b Correlation between PB CFC-DC (data from the DC assay) and CFC-GM (data from the Mix-assay) with Numbers of $CD34^+$ (A) and $AC133^+$ Cells (B) at Steady State and during Mobilization in 10 Patients.



A

Pearson correlation coefficients, CFC-DC vs CD34, r=0.769; CFC-GM vs CD34, r=0.839 (p = <0.001 for both)



B

Pearson correlation coefficients, CFC-DC vs AC133, r = 0.773; CFC-GM vs CD34, r = 0.796 (p = <0.001 for both). All data from the kinetic studies (Figs 5.1 and 5.2) are included. Values below the level of detection of the assay (i.e. no events observed) were assigned 10^{-1}(i.e. 0.1) for the purposes of presentation.

5.4 Discussion

This is the first report, which demonstrates clearly the pattern of DC progenitor mobilization in cancer patients following conventional dose chemotherapy and G-CSF. Several studies have shown DC numbers to increase following administration of myelosuppressive chemotherapy (Reid et al. 1990), r-HGFs (G-CSF, GM-CSF, IL-4, Flt-3L) (Gasparetto et al. 1999; Maraskovsky et al. 2000; Roth et al. 2000) or both (Avigan et al. 1999; Siena et al. 1995). Hart and colleagues assayed total steady state DC in healthy adults using the CMRF44 MoAb and determined the normal range for blood DC to be 3-17 $x10^3$/ ml PB. Although DC numbers rose acutely after surgical stress they declined in those patients mobilized with cyclophosphamide and G-CSF and were low when the PB $CD34^+$ cell count was at its highest (Fearnley et al. 1999; Hart et al. 1999). This finding is different from the observations of this study and other published data. CMRF44 is an activation antigen expressed on DC after *in vitro* culture (Fearnley et al. 1997) and it is possible that its expression is down regulated in blood DC derived from progenitors mobilized with cytotoxic chemotherapy and G-CSF.

Proper comparison of the numbers of clonogenic DC progenitors with those reported in the above studies is impossible because the patient populations and mobilization protocols were different. More importantly most studies (Avigan et al. 1999; Fearnley et al. 1999; Gasparetto et al. 1999; Maraskovsky et al. 2000; Roth et al. 2000) did not assay clonogenic DC. Reid and colleagues (Reid et al. 1990) who first showed the existence of a common clonogenic precursor for DC and macrophages enumerated DC progenitors in methylcellulose culture using non-adherent MNC. In steady state PB and after mobilization with chemotherapy alone, the mean peak values reported by Reid and colleagues (Reid et al. 1990) for CFC-DC

were 3.7 and 35.7 respectively per 10^5 cultured cells. As recovery of non-adherent cells can be as low as 50% of the total cell input while recovery of progenitors is almost 100% (Coutinho et al. 1993) the above figures are comparable to the numbers obtained in our study 1.3 in steady state PB and 18.1 in mobilized PB (data from fig 5.3b). Using the above mobilization protocols, which include administration of G-CSF, the frequency of CFC-DC might be expected to be lower as the production of neutrophils would be higher. The only other investigators who assayed DC progenitors were Siena and co-workers (Siena et al. 1995). They quantified DC progenitors in LP of cancer patients mobilized with high-dose cyclophosphamide, G-CSF and IL-3. They found the number of DC progenitors in LP to be higher than that obtained in this study (19.8 vs 4.7 $x10^4$/ kg respectively). Although the mobilization protocol and the assay method employed by them were different, they used mobilized PB $CD34^+$ cells and the same cytokine combination (as this study) *in vitro* plus Flt-3L (Siena et al. 1995). The addition of Flt-3L enhanced the generation of GM-CSF and TNF-α-dependent DC by 2-fold (Siena et al. 1995). One could speculate that this, combined with the 2-fold higher content of $CD34^+$ cells obtained in the LP -after mobilization with high-dose cyclophosphamide, G-CSF and IL-3- accounted for the 4-fold higher numbers of DC progenitors. Using limiting dilution analysis they estimated that the frequency (mean) of CFC-DC in LP was 1 DC progenitor/ 89 $CD34^+$ cells (95% CI = 1/75- 1/112). The corresponding figure obtained from 20 LP examined in this study was similar, 1 CFC-DC/ 84 $CD34^+$ cells (95% CI = 1/60-1/142).

The mobilization kinetics of CFC-DC was similar for most patients. For 7/10 patients CFC-DC and CFC-GM numbers in PB declined below baseline levels following chemotherapy and rose sharply during the mobilization phase. This "dip" in

CFC numbers did not parallel the changes in $CD34^+$ cell numbers. In the 3 remaining patients however the large drop in CFC-DC and CFC-GM numbers in PB appeared to correlate better with changes in the number of $AC133^+$ cells than $CD34^+$ cells. The $AC133^+$ subset of $CD34^+$ cells contains the majority of committed clonogenic GM and DC progenitors (de Wynter et al. 1998; Decatris et al. 1999). The increase in the percentage of $AC133^+$ cells as a fraction of the $CD34^+$ population from 37% at steady state PB to 74% during mobilization (statistically significant $p=0.001$) would suggest that AC133 might be a better predictor of GM and DC progenitor release. This needs to be tested in a larger study. There are no published data on the numbers of circulating $AC133^+$ cells in PB at steady state. However as the AC133 antigen is expressed on a subset of $CD34^+$ cells (Yin et al. 1997) and in normal BM this figure is 36% (de Wynter et al. 1998) these data confirm that the proportion of AC133 cells within the CD34 population in PB at steady state is almost identical to that in NBM.

The increase in PB CFC-GM was 25-fold in agreement with previous studies. These have reported increases in PB CFC-GM between 5-fold and 130-fold (Haas et al. 1993; Liu et al. 1993; Pettengell et al. 1993a; Weaver et al. 1996) in cancer patients mobilized with chemotherapy and G-CSF. The fold CFC-GM increase was in the lower end of this range and this is likely to be related to previous chemotherapy that some patients received. Separate analysis of 9 LP from 6 patients who were heavily pre-treated (median 6 cycles of chemotherapy) showed that the mean and median numbers of CFC, $CD34^+$ and $AC133^+$ cells were significantly lower than the corresponding values for patients treated with 1 chemotherapy cycle (data not shown). This is in agreement with published data (Brugger et al. 1992; Haas et al. 1994).

Mobilization of HPC with conventional-dose chemotherapy and G-CSF avoids the use of high-dose cyclophosphamide (4-7 gm/ m^2), which has been used either alone

(Kotasek et al. 1992; Rowlings et al. 1992; To et al. 1989) or in combination with HGFs (Schwartzberg et al. 1992). Although nearly all conventional-dose chemotherapy regimens have side effects, high-dose cyclophosphamide has a high incidence of toxicity (Jagannath et al. 1992; Kotasek et al. 1992). In one study as many as 44-100% of patients needed hospitalisation because of sepsis and a 3% mortality rate was reported (Rowlings et al. 1992). The other advantage of using conventional dose chemotherapy is that CFC-DC mobilization could be combined with tumour cytoreductive protocols specific for different cancers. This would be important in the adjuvant therapy setting or in treatment of minimal residual disease after chemotherapy; for example autologous CFC-DC could be collected following mobilization with adjuvant chemotherapy (and G-CSF); after pulsing with synthetic peptides or proteins derived from known tumour associated antigens (Brossart et al. 2001) they could used for vaccination after completion of chemotherapy.

Maraskovsky and colleagues (Maraskovsky et al. 2000) studied the expansion of DC subsets distinguished by the expression of CD11c, in healthy individuals using FLT-3L. FLT-3L has been shown to expand the numbers of DC *in vivo* in mice (Maraskovsky et al. 1996) and *in vitro* (Siena et al. 1995) or *in vivo* in humans (Maraskovsky et al. 2000). Using flow cytometric analysis the $CD11c^+$ subset (i.e. myeloid DC) was found to increase a mean 44-fold from baseline after 21 days of FLT-3L administration; the corresponding increase for the $CD11c^-$ fraction was 12-fold. These increases are of the same magnitude as those observed in this study for CFC-DC (Fig 5.3b). The relative fractions of precursors of CD11c positive and negative cells are unknown in our study; however the *in vitro* cytokine conditions used would favour differentiation of myeloid DC progenitors. This group would include $CD11c^+$ cells, which also express other myeloid markers like CD33

(Maraskovsky et al. 2000). In another study Gazitt and co-workers demonstrated 3- to 10-fold increases in $CD80^+$ DC precursors mobilized with cyclophosphamide and GM-CSF (Gazitt et al. 1999). Although the DC in that study would be expected to be less primitive than the CFC-DC we assayed and the mobilization protocols differ it is interesting that the fold increases over baseline are of the same magnitude.

The optimum number of DC required for cancer immunotherapy is unknown; in reported phase II studies it has varied depending on the protocol. Hsu and colleagues (Hsu et al. 1996) used a median 5 x 10^6 DC per immunization to vaccinate patients with lymphoma. According to their protocol, 4 treatments would require a median 20 x10^6 DC. These numbers of DC were sufficient to elicit measurable cellular immune responses against the tumour with clinical responses in 3 out of 4 patients. Nestle and colleagues (Nestle et al. 1998) achieved objective tumour responses using 6x10^6 peptide- or tumour lysate-pulsed DC in patients with metastatic melanoma. The LP in our study contained a median 3.8 x 10^4 CFC-DC/ kg, which in a 70kg individual would equate to a total 2.7x 10^6 DC progenitors. Many of the studies in which DC have been generated from $CD34^+$ cells have reported a 10-40 fold expansion *in vitro* (Caux et al. 1992; Siena et al. 1995; Timmerman and Levy 1999); this would yield 27-108 x 10^6 $CD34^+$-derived DC. These should be sufficient for vaccination if the above protocols were used. Moreover FLT-3L could be used to increase further DC yield from $CD34^+$ cells. The kinetics of *in vivo* expansion of the $CD11c^+$ subset of DC –which also co-expressed CD33 suggesting myeloid origin- differed from those of CFC-DC mobilization in that peak numbers were seen on day 9 (Maraskovsky et al. 2000), i.e. two days sooner than peak progenitor numbers in PB in our study. This may be due to the different patient population studied (i.e. healthy volunteers who mobilize more quickly) or the fact that FLT-3L mobilizes DC progenitors faster than

chemotherapy and G-CSF. Future work should involve a direct comparison of CFC-DC yields between FLT-3L ± chemotherapy versus G-CSF combined with chemotherapy as well as kinetic studies looking at the mobilization of the clonogenic myeloid and lymphoid CFC-DC. This may be useful in establishing the optimum time of aphaeresis for such patients as the 2 classes of progenitors may mobilize differently. Subgroup analysis comparing cell and CFC numbers between patients mobilized with cisplatin-based chemotherapy (BEP or VIP, n = 9) and anthracycline ± taxane-based chemotherapy (FEC or AT or CHOP, n = 6) showed a trend for higher yields in the former group (data not shown). However the differences were not statistically significant (p values > 0.05).

The importance of collecting large numbers of GM progenitors for haemopoietic reconstitution is known (To and Juttner 1993). Rapid engraftment following myeloablative chemotherapy and PBPC transplantation might not take place below a minimum threshold of 15-20 $x10^4$ CFC-GM/ kg (To et al. 1997). Similarly it may be important for haemopoietic autografts to contain a minimum number of DC progenitors to facilitate reconstitution of the immune system after myeloablative therapy. The importance of DC (and their precursors) cannot be underestimated because of their crucial role in antigen processing / presentation and control over B and T lymphocyte function (Banchereau and Steinman 1998). The role of CFC-DC in the early days post PBSCT when patients develop transplant related complications (mainly in the allogeneic setting) may prove to be significant because the *in vivo* cytokine conditions in such patients are often characterized by high levels of pro-inflammatory cytokines like TNF-α / IL-1 (Holler et al. 1990). These cytokine conditions at least *in vitro,* favour DC maturation from $CD34^+$ progenitors. This would be particularly important in the early days of engraftment before monocytes

reach appreciable numbers in PB. It will therefore require further work to establish the clinical significance of quantitative differences of CFC-DC in autologous and allogeneic stem cell grafts.

CFC-DC and CFC-GM are derived from a common CFC-GMDC myeloid precursor (Young 1999b). Consequently the strong positive correlation between CFC-GM and CFC-DC (each assayed independently with a specific clonogenic assay) with $CD34^+$ and $AC133^+$ cells strengthens further the validity of the DC assay. $AC133^+$ cells, which were also $CD34^+$, contained the majority of CFC-GM (Yin et al. 1997) and CFC-DC (Decatris et al. 1999). However the correlation of progenitors with AC133 cell numbers was not greater than with CD34 cell numbers, as might have been expected. The most likely explanation is that the majority of $CD34^+$ cells (~80%) were also $AC133^+$; so a positive correlation of CFC-GM/DC with CD34 cell numbers would be reflected to the same extent in a comparison with AC133 cell numbers.

5.5 Conclusions

The following conclusions can be drawn so far:

- In patients with solid tumours myeloid DC progenitor mobilization can be easily achieved using conventional dose chemotherapy and G-CSF. This approach is simpler, can be incorporated into the cytoreductive part of anticancer therapy, obviates the need to use IL-3 in addition to G-CSF and avoids the toxicity of high dose cyclophosphamide.
- The optimum time for collection of such progenitors is when the WCC rises exponentially from nadir (10 days after the end of chemotherapy). This is in agreement with previous work done on the mobilization of CFC-GM (Pettengell

et al. 1993a) and is not surprising as both the CFC-DC and CFC-GM share a common G-M-DC progenitor (Caux and Banchereau 1996).

- Finally the assay of DC progenitors showed that their numbers in PB correlated with the numbers of $CD34^+$ and $AC133^+$ cells although further work is required to see if monitoring the expression of the AC133 antigen is superior to CD34 in predicting more precisely the release into PB of G-M-DC committed progenitors.

Chapter 6

Final Discussion-Future Directions

The data presented thus far show that myeloid DC progenitors, i.e. GM-CSF and TNF-α dependent CFC-DC, can be assayed like other HPC in semi-solid culture. The linearity, dose response experiments, light microscopy and immunocytochemistry results have validated the clonogenic assay. $CD34^+$ cells cultured in the CFC-DC assay with r-HGFs develop into DC. Functionally DC development depends on the combined presence of GM-CSF and TNF-α. This confirms earlier work (Caux et al. 1992; Santiago-Schwarz et al. 1992; Young et al. 1995). The phenotypic characterization included dendritic morphology, the large lobulated nucleus and the expression of 3 important DC markers. CD83 a member of the immunoglobulin superfamily has unknown function but is expressed on mature DC (Zhou and Tedder 1995a; Zhou and Tedder 1996). The intensity of CD83 expression was variable and this, combined with the varied morphology of DC would suggest asynchronous maturation *in vitro* consistent with previous observations (Young et al. 1995). HLA-DP-DR is an important molecule involved in antigen presentation and this together with the costimulatory molecules CD86 -and CD80- are up regulated in mature DC (Hart 1997). Although functional assays to measure the immunostimulatory capacity of cultured DC have not been done, the strong expression of both, HLA class II antigens and CD86 implies that DC cultured with this method would stimulate the proliferation of autologous and allogeneic T cells *in vitro*. Several investigators who used this clonogenic assay have confirmed this (Ratta et al. 1998; Reid et al. 1990; Young et al. 1995).

$AC133^+$ cells within the $CD34^+$ population were enriched for CFC-DC and CFC-GM, thus confirming earlier work done using liquid culture experiments and clonogenic assays (de Wynter et al. 1998; Decatris et al. 1999; Yin et al. 1997). The mobilization kinetics of CFC-DC showed that DC progenitors behave, as expected, like other myeloid progenitors but more importantly they confirmed for the first time the optimum time window for leukaphaeresis of DC progenitors. This is obviously important for the design of immunotherapy protocols where large numbers of DC must be generated from $CD34^+$ cells for vaccination.

The number of DC required for a clinically useful immune response is unknown although from animal data it is thought to be 10^7-10^8 (Morse and Lyerly 1998). Based on the author's results and with a modest 20-fold expansion of $CD34^+$ cells it should be possible to obtain $6x10^7$ DC (please see section 5.4). The best source of haemopoietic cells to generate DC for use in cancer vaccines is currently unknown; however the use of $CD34^+$ HPC may be preferable to Monocytes. Mobilization of CD34 cells can be incorporated into the anticancer therapy of patients. In addition the use of $CD34^+$ cells offers scope for genetic modification to enhance the Ag-presenting capacity of the mature DC.

The assay of CFC-DC in all the sources of HPC currently in clinical use is an important starting point for future work in the field of stem cell transplantation. This also applies to other pathological states such as cancer, infection, autoimmune disease and solid organ transplantation/ organ rejection. DC are pivotal in the control of immunity (Banchereau and Steinman 1998; Steinman 1991) and are thought to play a key role in immunosurveillance against cancer (Timmerman and Levy 1999). In the same way that $1x10^6$ (per ml PB) circulating neutrophils are critical in halting the establishment of bacterial and fungal infection, the numbers of DC in PB are likely to

be important in generating strong antitumour responses against circulating malignant cells and or established tumours. Therefore the numbers of DC progenitors may be one of the essential requirements for an effective response; others being qualitative parameters like the functional capability of the DC and a cytokine milieu that favours their production, differentiation and maturation and not one that is inhibitory.

If the number of PB CFC-DC correlates with the intensity of antitumour responses, this needs to be established in clinical trials of DC vaccination. A threshold level below which a biological event becomes more likely is seen *in vivo*. For example, neutrophil counts < $1.0x10^6$/ ml and platelet counts < $20x10^6$/ ml predispose to neutropenic sepsis and cerebral haemorrhage respectively while harvests containing less than 15-20 $x10^4$/ kg CFC-GM or 2 $x10^6$/ kg $CD34^+$ cells reduce the chances of successful engraftment within 2 weeks post stem cell transplant (To et al. 1997). Assaying CFC-DC in different diseases may identify a threshold level essential for an enhanced immune response in susceptible individuals (e.g. with malignancy in the setting of minimal residual disease or patients on immunosuppressive therapy). This target threshold level could be reached using DC mobilization strategies or infusions of DC prepared *ex vivo* from $CD34^+$ progenitors.

The results in chapter 4 demonstrated, like other researchers, that umbilical CB is enriched for myeloid progenitors compared to BM (Broxmeyer 1998; Gluckman et al. 1999; Mayani and Lansdorp 1998). Despite however the higher numbers of DC progenitors/ 1,000 $CD34^+$ cells in CB, the fetus is immunologically naive. DC function in the neonate is reduced, in sharp contrast to adult PB (Petty and Hunt 1998). This therefore raises questions about the possible use of CB DC progenitors to reduce allograft rejection in solid organ transplantation. The Ag presenting capacity of CB DC may be restricted because of reduced expression of MHC antigens; this may

be due to failure of production of GM-CSF, IL-4 or TNF-α in adequate amounts (Petty and Hunt 1998). As a result the DC do not mature in the fetus and cannot stimulate T lymphocytes. Studies on the intracellular cytokine profile of CB and adult PB lymphocytes have shown that the former produce less IL-2, IL-4, IFN-γ and TNF-α (Chalmers et al. 1998). Of these cytokines, TNF-α is vital for inducing DC maturation. The importance of TNF-α in enhancing DC function and numbers has been shown in several studies (Caux et al. 1992; Caux et al. 1996b; Chen et al. 1998; Szabolcs et al. 1995). Reduced production of critical cytokines and restricted DC maturation may therefore be one of the mechanisms to account for the immunological immaturity of CB. This however does not preclude their potential clinical use in immunotherapy.

DC progenitors deficient in costimulatory molecules have been used to induce tolerance in solid organ transplantation in preclinical studies (Thomson et al. 1995). Although hepatic DC progenitors have been shown to prolong pancreatic islet allograft survival (Rastellini et al. 1995) the main limitations are the scarcity of solid organ DC for clinical use and the logistics of human transplantation (e.g. narrow time window for marrow transplant before the organ transplant). However allogeneic CB is more widely available and CB-derived costimulatory molecule-low/ deficient DC progenitors given before solid organ transplant may induce T-cell tolerance and prolong allograft survival. This has already been tested successfully in mice (Fu et al. 1996).

The data on the enrichment of CFC-DC and CFC-GM within the $AC133^+$ cell fraction are supported by the expansion of DC using FLT-3L (Maraskovsky et al. 1996; Maraskovsky et al. 2000; Maraskovsky et al. 1998). FLT-3L binds the FLT-3 receptor tyrosine kinase that is expressed on the majority of BM $AC133^+$ cells

(Buhring et al. 1999), which also contain most CFC-DC. The function of the AC133 Ag -recently designated CD133 by the 7th Workshop and Conference on human Leucocyte Differentiation (Wuchter et al. 2001)- is as yet unknown. Work done so far suggests it may be an early HPC Ag and as such, play a role in HPC differentiation (Miraglia et al. 1997; Yin et al. 1997). It would be challenging to speculate that because of the marked dichotomy with respect to differentiation, between AC133 positive and negative cells, this Ag expression might confer a granulo-monocytic / dendritic cell differentiation potential. Conversely loss of AC133 expression may allow for erythroid/ lymphocyte differentiation. This notion is further supported by the fact that only 1.5% of $AC133^-$ cells express the myeloid marker CD33 while as many as 26% and 28% express the lymphoid markers CD10 and CD19 respectively (Buhring et al. 1999). It has been suggested that AC133 is the human homologue of the mouse kidney prominin a 115-kDa protein with a five transmembrane domain that is expressed on embryonic epithelia and neuroepithelial cells (Weigmann et al. 1997).

More recently it has been shown that $AC133^+$ cells from G-CSF mobilized PB have the capacity to differentiate *in vitro* into endothelial cells (Gehling et al. 2000). It has also been shown that mobilized PB contains a small (2%) but distinct population of circulating endothelial precursors (CEPs) which co express the VEGF receptor-2 (VEGFR-2) and AC133 (Peichev et al. 2000). In physiological conditions these CEPs may be involved in neo-angiogenesis, however it is possible malignant tumours may use such cells for neovascularization (Gehling et al. 2000). Therefore FLT-3L, which mobilizes $AC133^+$ cells must be used cautiously and only after further evaluation in clinical trials. The implications of this however may go beyond the use of FLT-3L. $CD34^+$ HPC mobilization with conventional chemotherapy and rh-G-CSF in this study resulted in the release into PB of 184,000 $CD34^+$ cells/ ml (at peak

mobilization). If 2% of these cells express VEGFR-2 (Peichev et al. 2000) then one would expect one ml of PB to contain ~3,680 putative CEPs. Assuming a blood volume of 4,000 ml then ~$15x10^6$ CEP could be mobilized into PB in total. The frequency of clonogenic cells has only been determined for CEPs from fetal liver (3%) (Peichev et al. 2000). The corresponding figure for CEPs from adult PB would be expected to be lower. However even if the frequency is estimated conservatively to be 1% this would still result in $15x10^4$ clonogenic CEPs. Although no evidence has published which supports that this occurs *in vivo,* it is conceivable that some of these 150,000 CEPs could migrate into sites of established micrometastatic disease and be involved in tumour neovascularization. AC133 is selectively expressed on subsets of CEPs but not on mature endothelial cells (Rafii 2000). It could therefore be used in conjunction with VEGFR-2 to isolate CEPs and test their role in tumour angiogenesis *in vivo* in animals.

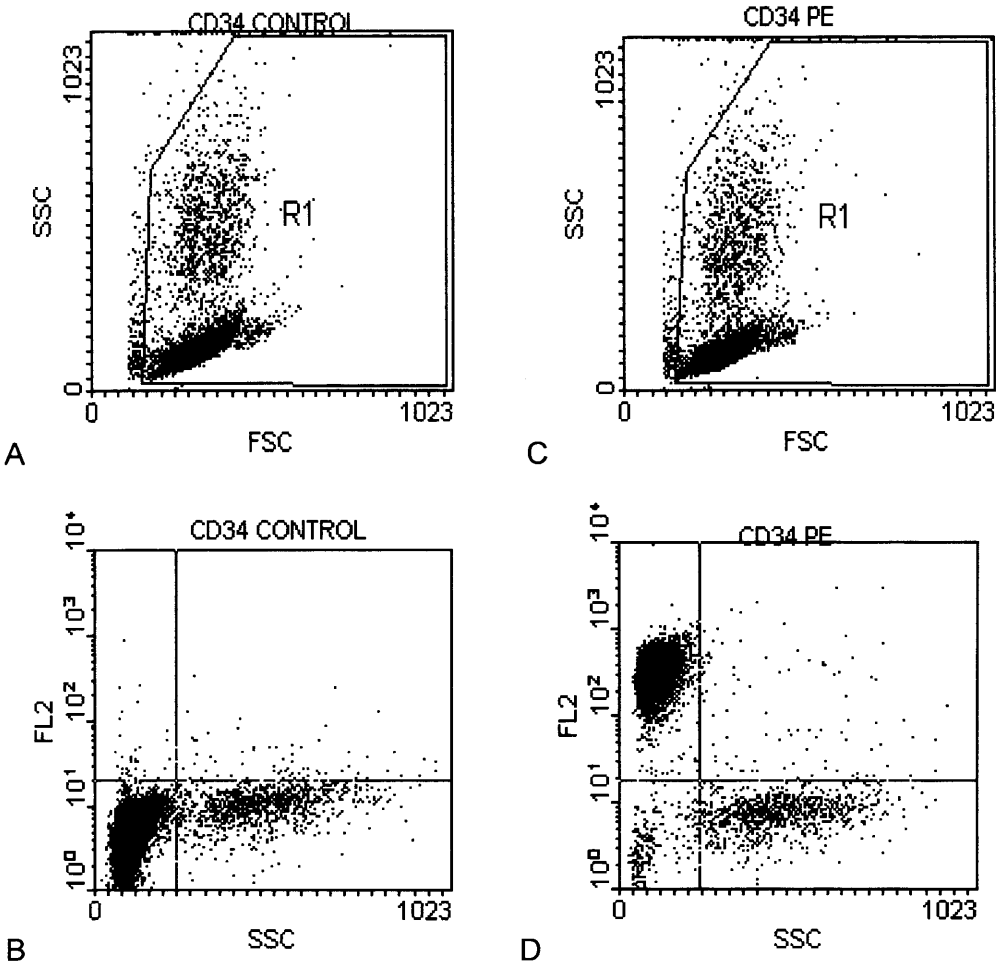
The kinetic studies confirmed that conventional dose chemotherapy and G-CSF can mobilize DC progenitors among the other progenitors released from the BM into PB. The release of large numbers of CFC simultaneously with the rapid rise in WCC is supported by earlier work on the mobilization of CFC-GM (Pettengell et al. 1993a). This can be used as a guide for starting the aphaeresis in centres where the results of CD34 analysis cannot be made available on the same day. New strategies for more effective CFC-DC mobilization with combinations of G-CSF plus SCF or G-CSF plus FLT-3L need to be tested. If these prove considerably more effective in mobilizing CFC-DC, they may obviate the need for a 3-hour aphaeresis.

The last 8-9 years have seen a remarkable advance in DC biology, as we are now able to generate these cells in sufficient numbers for experimental work and for clinical trials. DC vaccination is still in its infancy. Despite this, three early clinical

trials in lymphoma, (Hsu et al. 1996) advanced melanoma (Nestle et al. 1998) and more recently in metastatic renal cell carcinoma (Kugler et al. 2000) have achieved astonishing results with complete/ partial responses in some patients. Steïnman's methodical work that led to the identification and characterisation of the dendritic cell and the work of many others have started to pay off. It is hoped that the work in this thesis will contribute to our knowledge on the dendritic cell for years to come.

APPENDIX A: Calculation of purity of $CD34^+$ cells after Minimacs separation

A FACS profile of isolated human PB $CD34^+$ cells labelled with CD34-PE conjugated MoAb.



CD34-PE labelled cells are plotted with the side scatter (SSC) features against the forward scatter (FSC) features (C). Events gated into R1 (excludes red cells and debris) are displayed on a second dot plot (D) with CD34-PE fluorescence (FL2) against SSC. The % of cells in the upper left quadrant of plot D is the % of $CD34^+$ cells with the SSC characteristics of HPC. The same gating strategy is applied for the control sample (plots A, B) and the % of events in the upper left quadrant (background fluorescence, plot B) is subtracted from that in plot D. The control quadrants were always set first to ensure 99% of the gated events were CD34neg.

Statistics tables based on the above dot plots:

File: SCAN.007- Sample ID: CD34 CONTROL
Gates: R1
Gated Events: 19738
Quad Stats
Empty(Lin) vs Empty(Log)
Quadrant x,y: 236,332

Quad	X-Mean	Y-Mean	Events	%Total	**%Gated**
1 UL	116.1	41.0	42	0.21	0.21
2 UR	539.7	33.6	82	0.41	0.42
3 LL	100.4	4.7	18615	93.08	**94.31**†
4 LR	476.8	9.3	999	5.00	**5.06**†

† > 99% events (94.31% + 5.06% in the lower left (LL) and lower right (LR) quadrants respectively -dot plot B-) from the control sample are CD34neg.

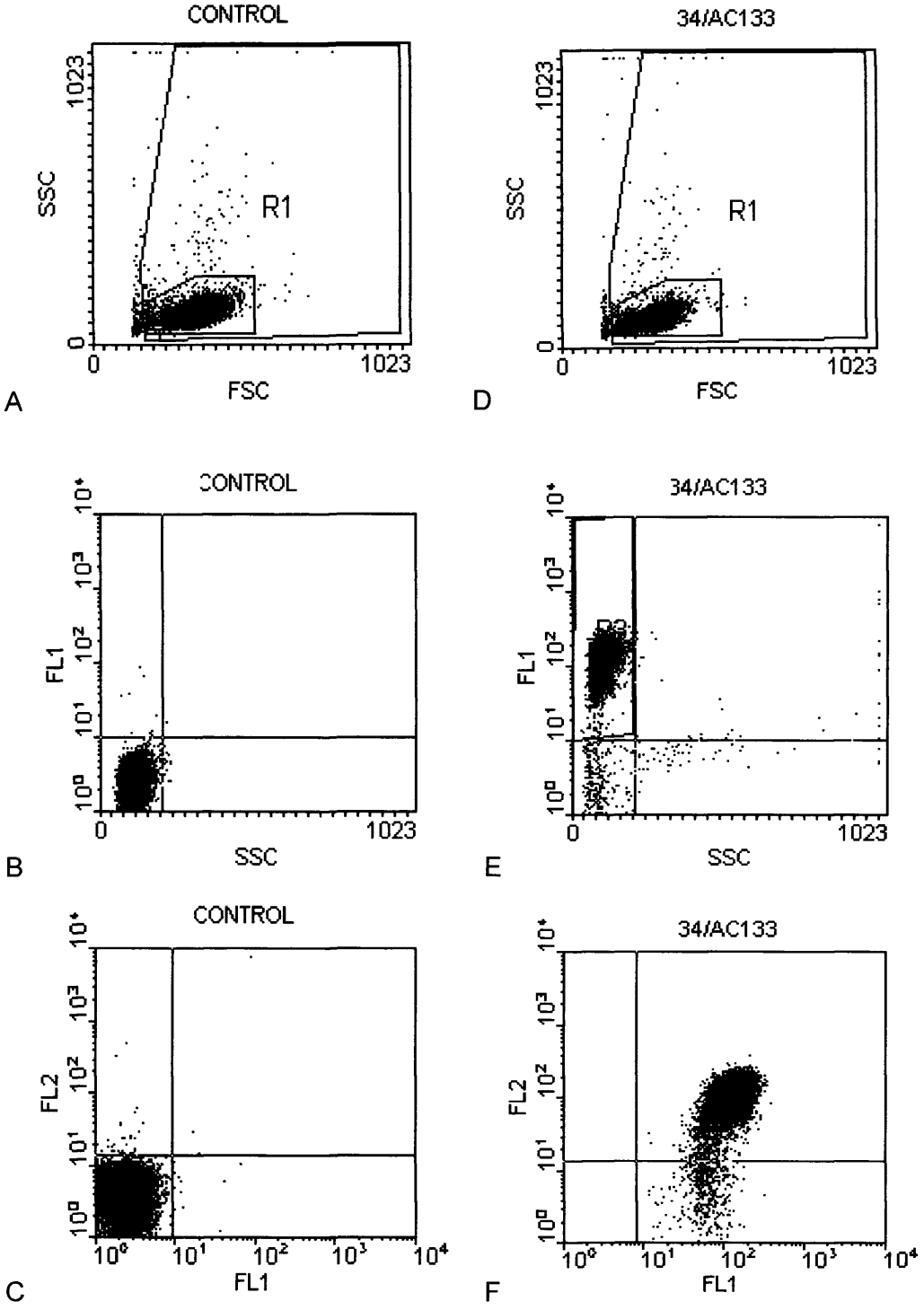
File: SCAN.008- Sample ID: CD34 PE
Gates: R1
Gated Events: 19713
Quad Stats
Empty(Lin) vs Empty(Log)
Quadrant x,y: 232,324

Quad	X-Mean	Y-Mean	Events	%Total	**%Gated**
1 UL	94.4	305.0	18446	92.23	**93.57**†
2 UR	477.3	137.6	102	0.51	0.52
3 LL	97.6	3.1	266	1.33	1.35
4 LR	458.4	7.3	899	4.50	4.56

† Percentage purity of $^{\dagger}CD34^{+}$ cells = 93.57% (plot D) minus background fluorescence from corresponding quadrant in dot plot B (0.21%).

APPENDIX B: Calculation of AC133$^{+/-}$ percentages within the CD34^{+} cells

FACS profile of isolated human PB CD34^{+} cells labelled with CD34-FITC and AC133-PE conjugated MoAbs during FACS sort.



CD34-FITC and AC133-PE labelled cells are plotted with the SSC features against the FSC features (D). Events gated into R2 (lymphocyte gate; i.e. the smaller polygon within R1 in plots A and D) are displayed on a second dot plot (E) with CD34-FITC fluorescence (FL1) against SSC. Events within the upper left quadrant (plot E) are gated into region R3. Events within R2 and R3 are displayed in dot plot F with AC133-PE fluorescence (FL2) against CD34-FITC fluorescence (FL1). The relative percentages of $CD34^+AC133^+$ and $CD34^+AC133^-$ cells are given by the % of cells in the upper and lower right quadrants respectively of plot F. The control quadrants were always set first to ensure 99% of the gated events were $CD34^-AC133^-$ (plot C). The same gating strategy was applied for determining the percentages of $CD33^+$ and $CD33^-$ cells within the CD34 population.

Statistics tables based on the above dot plots:

File: Data.011- Sample ID: CONTROL
Gates: *R2
Gated Events: 19287
Quad Stats
Empty(Log) vs Empty(Log)
Quadrant x,y: 252,296

Quad	X-Mean	Y-Mean	Events	%Total	**%Gated**
1 UL	2.3	23.8	57	0.29	0.30
2 UR	30.2	95.8	7	0.04	0.04
3 **LL**	2.4	3.7	19199	96.00	**99.54**†
4 LR	15.4	4.6	24	0.12	0.12

† > 99% events from the control sample in the lower left (LL) quadrant (dot plot C)

File: Data.013- Sample ID: 34/AC133
Gates: *R2*R3
Gated Events: 19110
Quad Stats
Empty(Log) vs Empty(Log)
Quadrant x,y: 236,292

Quad	X-Mean	Y-Mean	Events	%Total	**%Gated**
1 UL	0.0	0.0	0	0.00	0.00
2 **UR**	125.5	99.7	18107	90.54	**94.75**†
3 LL	0.0	0.0	0	0.00	0.00
4 **LR**	58.3	5.2	1003	5.02	**5.25**‡

Percentage of $^{\dagger}CD34^+AC133^+$ cells = 94.75% (plot F) minus background fluorescence from corresponding quadrant in dot plot C (same applies for $^{\ddagger}CD34^+AC133^-$ cells).

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Publications

List of publications arising from this work

Decatris, M. P., de Wynter, E. A., Wilkinson, P. M., and Testa, N. G. "Mobilization Kinetics of Myeloid Dendritic Cell Progenitors in Patients with Solid Tumours." Late Breaking Abstracts of the 1999 Annual Meeting of the International Society for Experimental Haematology. Poster Presentation.

Articles in preparation

Decatris, M. P., de Wynter, E. A., Wilkinson, P. M., Welch, R. S., and Testa, N. G. "A quantitative study of myeloid dendritic cell progenitors and their mobilization kinetics in patients with solid tumours."

Decatris, M. P., de Wynter, E. A., Wilkinson, P. M. and Testa, N. G., "A comparative quantitative study of human myeloid dendritic cell progenitors in bone marrow, cord blood and mobilized peripheral blood."

LBA008

Poster Session I
Sunday, July 11, 1999
The Monte Carlo Convention Center and Auditorium (C.C.A.M.) - Loews

MOBILISATION KINETICS OF MYELOID DENDRITIC CELL PROGENITORS IN PATIENTS WITH SOLID TUMOURS.

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We assayed myeloid Dendritic cell (DC) progenitors in the leucaphaeresis products (LP) of 13 patients with solid tumours and studied the kinetics of their mobilization to establish the optimum time for aphaeresis of clonogenic DC progenitors (CFC-DC).

Haemopoietic progenitors were mobilised with conventional dose chemotherapy and lenograstim administered at 5μg/kg/day in patients with metastatic teratoma (n=8) and 263μg/ day in patients with breast cancer (n=5). The numbers of granulocyte-macrophage (CFC-GM) and erythroid progenitors (BFU-e), CD34+ and AC133+ cells were also determined in peripheral blood (PB) and LP. For kinetic studies 8 patients had PB taken at 1-3 day intervals during recovery of the white cell count (WCC).

The median numbers (range) of progenitors collected in 17 LP (4 patients had 2 collections) were; CFC-DC 3.8 $x10^4$/kg (0.5-12.5), CFC-GM 15.4 $x10^4$/kg (6.0-74.4), BFU-e 26.3 $x10^4$/kg (7.4.0-278.0). The corresponding numbers of cells collected were; MNC 3.5 $x10^8$/kg (1.6-7.8), CD34+ 6. $x10^6$/kg (1.6-20.3) and AC133+ 4.8 $x10^6$/kg (1.3-16.8). The kinetics of mobilization of DC, GM and erythroid progenitors followed a similar pattern, with maximal numbers of release during the 2-3 day period that the WCC rose rapidly from 3 $x10^9$/L.

We conclude that the optimum time for aphaeresis of myeloid DC progenitors in patients with solid tumours is the same as for GM and erythroid progenitor collection.