Haemopoiesis, Leukaemia & Imatinib: c-fms, a Novel Target for Small Molecule Inhibitor Therapy

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A thesis submitted to the University of Adelaide
in candidature for the degree of Doctor of Philosophy
September 2004

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Andrea Dewar

ACKNOWLEDGEMENTS

Thanking everyone for all their help during my PhD isn't an easy task, as there have been many people over the course of my studies that have gone out of their way to help. And I know that without their encouragement and support, I wouldn't have got this far.

First of all, I'd like to thank my supervisors Dr Kathleen Doherty and Dr Bruce Lyons. I don't doubt that by being a determined student who was keen to get finished two weeks after starting has driven you crazy at times! Thank you for your ideas, patience, guidance and stories of the good old days. As a lasting legacy of your supervision, I'm sure the terms "proactive" and "interesting" will be sounding in my ears forever.

Thank you to Associate Professor Tim Hughes for all your valuable ideas during meetings and for allowing me to carry out my PhD in your lab. Thank you also to other members of the Melissa White Laboratory - Deb White, Dr Pongtep Viboonjuntra and Verity Saunders - for all your willing help in the lab (even when you've already told me once before, Verity!) and for putting up with my rants and raves when experiments went pear-shaped. A special thank you goes to Bernadette Miller for keeping me entertained and sane, for being an eager and enthusiastic assistant to a bossy boss, and for always being there to help me out and listen when I needed it the most. Thank you also to Dr Rachel Gibson who left the MWL mid-way through my studies, but still went out of her way to do anything for me and dropped by regularly to keep me inspired and say hello.

When all else failed, or even when I didn't have an excuse, I always managed to find smiling faces, help and a sounding board around the corner in the Matthew Roberts Laboratory. Thank you to Dr Andrew Zannettino and Dr Stan Gronthos for always having time to help me out and for providing me with invaluable suggestions, advice and humour. Thank you to Amanda Farrugia, Angela Kortesidis and Dr Betty Pan for always having a spare chair for me in the lab or office, for allowing me to run off with equipment and for listening to all my stories. Your friendships have meant a lot to me and have made the past three years a lot easier than what they would otherwise have been.

Members of the Molecular Regulation Laboratory have also provided me with an enormous amount of advice and support, given answers to my endless questions, and regularly dropped by to check that things were going ok. Thank you in particular to Andrew Fotia, Dr Stuart Read, Dr Dimitrious Kacourous and Dr Linda Sherwin. An extra thank you also goes to Andrew Fotia for keeping me laughing with all his office humour, and for telling stories that were always very believable.

Thank you to Tony Cambareri for providing me with an enormous amount of advice, happily answering all my questions and never complaining when I came back to ask even more! Your patience and willingness to help is greatly appreciated.

There are many other people in the Hanson Institute that have helped me during the course of my studies - thank you to Dr Haylee Ramshaw for help with colony assays, and Ghafar xxxx for confocal microscopy. Thank you to Alan Bishop and Sandy Macintyre for your expertise in flow cytometric cell sorting, happily finding spare time to squeeze in a last-minute experiment and for dropping by to see how things were going. I would also like to thank the Australian Red Cross Blood Service, South Australia for providing me with buffy coats whenever I needed them.

Endless support for my studies has also come from my family and friends - even though they never understood what I was up to, they asked, listened and cared. Some even went the extra distance and attempted to pick up some science jargon - so to them, I give another aliquot of thanks.

Thank you to Mum and Dad who aside from being great parents, are also great friends. I am very fortunate to be part of such a supportive and loving family, and am extremely grateful for all your encouragement, for being there whenever I needed you and for your phone calls to break up the tedium of writing. Thank you also to my brother Mark, who gets a mention simply because he wanted to be mentioned.

The biggest thank you goes to my husband, Michael. You have provided me with an endless amount of love, encouragement and support, and my PhD would have been even

tougher without you by my side. You are an extremely wise person, and your perspective has always provided me with guidance and the inspiration to keep going with a smile on my face. And as a well known song sums it up, "I want to thank you for giving me the best day of my life…. to be with you is having the best day of my life".

Lastly, I would like to thank Professor To for allowing me to undertake my studies in the department of haematology at the Hanson Institute, and the University of Adelaide and Lions Medical Research Foundation for providing financial support during my studies.

“Obstacles cannot crush me;
every obstacle yields to stern resolve”

Leonardo da Vinci

“Don’t wait for a light to appear at the end of the tunnel;
stride down there and light the bloody thing yourself!”

Sara Henderson

ABSTRACT

Understanding the factors that regulate the growth and differentiation of haemopoietic stem cells (HSC) remains a major challenge. In this study, the proliferation and differentiation of $CD34^+$ cells from normal donors and chronic myeloid leukaemia (CML) patients was compared. The proliferation and entry of CML cells into the cell cycle was decreased relative to cells from normal donors, and greater heterogeneity in the phenotype of CML cells at the initiation of culture was observed. Analysis of phenotype concomitant with cell division also demonstrated that the differentiation of normal $CD34^+$ cells was consistent between donors, while marked variability was observed in the differentiation of $CD34^+$ cells from CML patients. This included expression of CD13, CD33, CD38 and HLA-DR, which were linked to cell division in normal but not CML cells.

The tyrosine kinase inhibitor, imatinib, is a novel drug displaying promising results in the treatment of CML by specifically inhibiting the growth of leukaemic cells. To examine whether myelosuppression observed in patients treated with imatinib may arise from inhibition of normal haemopoiesis, imatinib was added to colony assays established using cells from normal bone marrow. Suppression of monocyte/macrophage growth, but not that of eosinophils or neutrophils, was observed at therapeutic concentrations of imatinib. Inhibition of monocytic differentiation to macrophages was also observed and was associated with decreased functional capacity such as altered antigen uptake, production of proinflammatory cytokines and stimulation of responder cells.

The specific suppression of monocyte/macrophage differentiation and function was not due to blockade of tyrosine kinases known to be inhibited by imatinib and was consistent with an inhibition of the M-CSF/c-fms signalling pathway. This hypothesis was tested using a cell line that was dependent on M-CSF for growth and survival. Cell proliferation and phosphorylation of c-fms were inhibited at an IC_{50} of 1.9μM and 1.4μM imatinib respectively and this was not attributable to decreased c-fms expression. These important findings therefore identify c-fms as a further target of imatinib, and suggest that imatinib should be considered for treatment of diseases where c-fms is implicated. This includes breast and ovarian cancer and inflammatory conditions such as rheumatoid arthritis. Potential side effects resulting from imatinib treatment must also be considered.

ABBREVIATIONS

µg	Micro gram
µm	Micro metre
µM	Micro molar
4HGF	Four haemopoietic growth factors (IL-3, IL-6, G-CSF, GM-CSF)
5HGF	Five haemopoietic growth factors (IL-3, IL-6, G-CSF, GM-CSF, SCF)
ATP	Adenosine triphosphate
BM	Bone marrow
BMMNC	Bone marrow mononuclear cell
BSA	Bovine serum albumin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CM	Cultured monocyte
CML	Chronic myeloid leukaemia
CSF	Colony stimulating factor
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram
G-CSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumour
GM-CSF	Granulocyte-macrophage colony stimulating factor

HBSS	Hank’s balanced salt solution
HLA	Human leukocyte antigen
HSC	Haemopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove’s modification of Dulbecco’s medium
LPS	Lipopolysaccharide
M	Molar
MACS	Magnetic activated cell sorting
M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
m	Murine
mg	Milli-gram
mL	Millilitre
MLR	Mixed lymphocyte reaction
mM	Milli molar
MNC	Mononuclear cell
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
Ph chromosome	Philadelphia chromosome
PI	Proliferation index
Rh	Recombinant human
RT	Room temperature
SCF	Stem cell factor
SDM	Serum deprived medium
TNF	Tumour necrosis factor
TPO	Thrombopoietin
v/v	Volume per volume
w/v	Weight per volume

PUBLICATIONS

1. Imatinib Inhibits the Tyrosine Kinase Activity of the Macrophage Colony Stimulating Factor Receptor c-fms at Clinically Relevant Concentrations. **AL Dewar**, AC Cambareri, AC Zannettino, BL Miller, KV Doherty, TP Hughes, AB Lyons. *Manuscript in preparation.*

2. Imatinib inhibits the functional capacity of cultured human monocytes. **AL Dewar**, KV Doherty, TP Hughes, AB Lyons. *Journal of Immunology & Cell Biology* (2004). *In Press.*

3. Imatinib inhibits the *in vitro* development of the monocyte/macrophage lineage from normal human bone marrow progenitors. **AL Dewar**, RM Domaschenz, KV Doherty, TP Hughes, AB Lyons. *Leukemia* (2003). 17(9):1713-1721.

4. Acquisition of immune function during the development of the Langerhans cell network in neonatal mice. **AL Dewar**, KV Doherty, GM. Woods, AB. Lyons, HK Muller. *Immunology* (2001). 103: 61-69.

5. Prevention of autoimmunity by induction of cutaneous tolerance. GM Woods, YP Chen, **AL Dewar**, KV Doherty, BH Toh, HK Muller. *Cell Immunol* (2001). 207(1):1-5.

THESIS AMENDMENTS

Corrections Relevant to Entire Thesis

- Error bars represent the SEM, and were derived from triplicate data points unless otherwise stated.
- Human genes should be written in capitals and italics. Proteins encoded by these genes should be written with the first letter in capital case, and should not be preceded by the "c-" prefix.

Chapter 1: Introduction

- Page 11, paragraph 1: the final sentence should be deleted as it does not follow.
- Page 14, paragraph 3: RAS is part of the MAPK pathway. The MAPK pathway is therefore not a further example of signal transduction pathways activated following ligand binding to the GM-CSF, IL-3 and IL-5 receptors.
- Page 26: The word "invariably" should be changed to read "usually", as there are many cases where CML patents remain in chronic phase for longer than 5 years.
- Page 28, first paragraph, final sentence: *Heistercamp et al (1990)* reference is incorrect. The reference should be *Daley et al (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome; Science. Feb 16; 247(4944): 824-30.*
- Page 31, 2nd paragraph: Reference to Deininger *et al* (1997) in statements that imatinib inhibits the growth of CML cells with minimal effects on normal haemopoiesis were omitted.
- Figure 1.1: LT-HSC is a "long-term" HSC. ST-HSC is a "short-term" HSC.

Chapter 2: Materials and Methods

- Page 46, 2.3.1: The final sentence in paragraph one should read: "Media *were* prewarmed...".
- Page 47, 2.3.1.1 & page 71, 2.5.5: The dilutions of IL-3 and GM-CSF that provided maximal cell growth were determined by titration using a factor dependent cell line.
- Page 71. 2.5.5: Use of the term selected means that the cells were selected for the ability to grow in M-CSF.

Chapter 3: Results Chapter 1

- Viability was determined following calculation of the percentage of cells with decreased FS/SS values.
- Surface markers were chosen which were expressed on specific myeloid lineages.
- Table 3.2: CML values were deemed significantly different from normal controls when p values calculated using ANOVA were less than 0.05.
- The data in Figures 3.13-16 are derived from the same experiment as Figures 3.5-3.8.
- Page 99, paragraph 1: "proliferative potential" should read "proliferation".

Chapter 4: Results Chapter 2

- Figure 4.2: The data was normalised relative to the 5HGF control, not the 4HGF control.
- Figure 4.9A (and elsewhere): Contrary to the statement in the legend, the marker tool was set on the 95th percentile of the fluorescence for the negative control.
- Figure 4.10: The morphology of the cells is not that of promyelocytes. The cells display an atypical morphology (excentric nucleus and a large, clear, vacuolated cytoplasm), however tristaining suggests the cells are immature myeloid cells.

Chapter 5: Results Chapter 3

Page 129, 5.2.3.2: The opsonin used was derived from purified rabbit polyclonal IgG antibodies that are specific for the zymosan particles. Phagocytosis of opsonsised zymosan particles was mediated by Fc and/or complement receptors.

Chapter 6: Results Chapter 4

Figures 6.6 and 6.7: "...infected with human c-fms..." should read "infected with a retrovirus encoding human FMS...".

Chapter 7: General Discussion

Page 152, paragraph 2: Abl is an intracellular tyrosine kinase, not a receptor tyrosine kinase.

Chapter 1:

INTRODUCTION

1.1 Introduction: The Process of Differentiation

Cellular differentiation typically involves a change from a primitive progenitor cell to a mature cell capable of effector function through the acquisition of specialised functions and morphologies that were previously absent. It is a tightly regulated process that involves the sequential and ordered activation or suppression of genes, and is controlled by a variety of mechanisms which include cell-cell contact, intrinsic factors such as transcription factors, and extrinsic factors such as cytokines.

With regard to differentiation within the haemopoietic system, primitive progenitor cells gradually become more restricted in their differentiation potential following a succession of symmetric and asymmetric divisions. Once these progenitor cells commit to a particular lineage, distinct gene expression patterns are maintained by transcription factors to determine the phenotype of the cell, and ensure that these cells are unable to change their fate decisions (Graf 2002).

All forms of cancer are characterised by a lack of normal control of cellular differentiation and proliferation. Leukaemias are a group of cancers developing within the haemopoietic system, and are associated with aberrations in the signalling pathways that control the differentiation and division of haemopoietic cells. As a consequence, there is a significant increase in the expansion of immature progenitor cells that fail to undergo a complete program of differentiation during later stages of the disease, and this can involve lymphoid (T and B cells), myeloid, erythroid, megakaryocytic and monocytic lineages (Faderl *et al.* 1999).

While some insight has been gained into the important role of transcription factors in haemopoiesis, it is still unclear how differentiation decisions are made and how aberrations in this process contribute to leukaemogenesis. A deeper understanding of the mechanisms behind haemopoietic stem cell (HSC) differentiation and leukaemia will potentially aid in the development of new therapeutics targeted at treating this haematological disease.

1.2 Haemopoiesis & Haemopoietic Stem Cells

Of all the stem cell systems, the molecular pathways of haemopoiesis are the most widely studied. Haemopoiesis is defined as the generation of the cellular elements of blood, and HSC are responsible for maintaining blood cell formation throughout the lifetime of an individual. This is due to the unique property of each HSC to sustain self-replication for approximately 50 cell divisions, which is sufficient to provide cells of the blood system for several life spans (Whetton and Graham 1999).

While the precise definition of a HSC is controversial and varies widely, it is accepted that a stem cell must exhibit both the capacity to give rise to more cells with identical proliferative and developmental capacities ("self-renew"), and generate differentiated progeny which are capable of long term reconstitution of the haemopoietic system. It is crucial for stem cells to exhibit self-renewal characteristics so that progeny capable of differentiating into multiple haemopoietic lineages such as lymphocytes, granulocytes, erythrocytes, and megakaryocytes are maintained long-term. In the steady state, the majority of stem cells are maintained in an inactive "quiescent" state, and only a small number of stem cells supply the haemopoietic system at any one time.

1.3 The Origin of Haemopoietic Stem Cells

1.3.1 Primitive & Definitive Haemopoiesis

In mammals haemopoiesis is divided into two main systems, the primitive embryonic system and the definitive system (for review, see Cumano and Godin 2001). During primitive haemopoiesis the first haemopoietic cells (large, nucleated erythroid cells) arise in the extraembryonic yolk sac and supply oxygen and nutrients to the developing tissues by circulating throughout the embryo (Marshall and Thrasher 2001). As embryonic development progresses, this primitive circulatory system is replaced by definitive haemopoiesis where the foetal liver, bone marrow, thymus and spleen are colonised by small nucleated erythrocytes. Unlike cells from the primitive system, precursor cells from the definitive system are dependent on erythropoietin for the final stages of differentiation and are capable of long term reconstitution of lethally irradiated adult recipients (Cumano and Godin 2001).

1.3.2 Formation of the Haemopoietic System

Initially it was believed that formation of the haemopoietic system began in the extraembryonic yolk sac by differentiation of the mesoderm and that this was followed by progressive migration of cells from the yolk sac to the liver and finally to the bone marrow. This assumption was based on the observation that the first haemopoietic cells identifiable in mice appear within the blood islands of the yolk sacs 7.5 days post fertilisation, and that most of these cells belong to the primitive erythroid lineage (reviewed in Cumano and Godin 2001).

More recently it has been suggested that the major source of embryonic stem cells is from within the embryo, rather than the liver being colonized by stem cells derived from the extraembryonic yolk sac. This theory arose following the isolation of haemopoietic precursor cells from an intraembryonic equivalent region, and the demonstration that yolk sac progenitors from avian embryos failed to contribute to definitive erythropoiesis (Cumano and Godin 2001). In mice this intraembryonic region is known as the aorta-gonad-mesonephros (AGM), and is derived from the para-aortic splanchnopleural mesoderm (P-Sp), which contains the dorsal aorta, putative gonadal ridge and mesonephros (reviewed in Marshall and Thrasher 2001). These haemopoietic precursor cells located in the AGM arise independently of yolk sac progenitors (Fuchs and Segre 2000) and are the first cells capable of long-term reconstitution of the haemopoietic compartment in lethally irradiated mice (Muller *et al.* 1994).

Cells residing in the embryonic AGM have been shown to express many haemopoiesis-associated markers including c-kit and the transcriptional regulators GATA-2, acute myeloid leukaemia (AML)-1 and stem cell leukaemia (SCL). The importance of these markers in both primitive and definitive haemopoiesis has been demonstrated using gene disruption studies. For example, GATA-2$^{-/-}$ embryos showed severe anaemia and only survived up until the onset of foetal liver haemopoiesis, while GATA-2$^{-/-}$ cells failed to contribute to sites of haemopoiesis (Tsai *et al.* 1994). In embryos with null mutations for the SCL gene, haemopoietic cells were absent from the yolk sac, and SCL$^{-/-}$ cells were incapable of colony formation, demonstrating a crucial role for SCL in the formation of yolk sac haemopoietic cells (Robb *et al.* 1995).

The current model for the formation of the haemopoietic system suggests that the first set of haemopoietic precursors develop in the yolk sac and provide a source of erythrocytes before the foetal liver begins active enucleated erythrocyte production at 11 days post fertilisation. A second set of haemopoietic precursors develops in the AGM and it is these cells that colonise the foetal liver and are ultimately responsible for adult haemopoiesis (Cumano and Godin 2001).

1.3.3 Homing of Haemopoietic Stem Cells to Appropriate Environments

Migration of HSC to the liver depends heavily on β1-integrin expression (Potocnik *et al.* 2000). The generation of β1-integrin-deficient HSC has demonstrated a vital role for this molecule in colonisation of appropriate environments such as the foetal liver, thymus spleen or bone marrow, but not in the differentiation of blood cells (Hirsch *et al.* 1996; Potocnik *et al.* 2000). For example, analysis of $\beta1^{-/-}$ chimeric embryos at various stages of gestation revealed the presence of $\beta1^{-/-}$ cells in the blood but not in the liver (Hirsch *et al.* 1996). The cellular content of blood cell colonies in cultures selected for β1-integrin deficient cells was not affected by the absence of β1-integrin (Hirsch *et al.* 1996), and the differentiation of $\beta1^{-/-}$ precursor cells into erythroid, myeloid and lymphoid cells *in vitro* was similar to that of $\beta1^{+/+}$ cells (Potocnik *et al.* 2000).

While in the foetal liver, some HSC differentiate to give rise to more restricted progenitor cells and, just before birth, migrate to the bone marrow where they remain for the lifetime of an individual supplying cells of the definitive haemopoietic system. The migration of HSC from the liver to the bone marrow is heavily dependent on the chemokine Stromal Derived Factor-1α (SDF-1α), which binds monogamously to its receptor CXCR4 (Tachibana *et al.* 1998; Zou *et al.* 1998; Whetton and Graham 1999). SDF-1 is secreted by bone marrow stromal cells, and expression of CXCR4 on HSC facilitates attraction of the HSC to the marrow microenvironment by means of a chemokine gradient (Kim and Broxmeyer 1998; Whetton and Graham 1999).

Once HSC colonise the bone marrow, retention of cells within marrow microenvironment is crucial. Bone marrow stromal cells express a range of extracellular matrix molecules

such as collagens I-VI and fibronectin, as well as cellular adhesion molecules (CAMs) such as ICAM-1 (Whetton and Spooncer 1998). Integrin receptors also play a crucial role in the interaction of HSC with stromal cells, with β1-integrin receptors involved in localisation and anchoring of HSC to appropriate environments as well as HSC trafficking between the bone marrow and circulation (Verfaillie *et al.* 1991; Williams *et al.* 1991; Teixido *et al.* 1992; Verfaillie *et al.* 1994; Papayannopoulou *et al.* 1995).

1.4 Regulation of Stem Cell Fate

Once HSC have colonised the bone marrow they must perform two vital roles: maintain a pool of HSC capable of self-renewal divisions, and supply mature cells of multiple haemopoietic lineages. In order to perform these roles, HSC are faced with four options: programmed cell death, quiescence, differentiation and self-renewal.

Recent evidence suggests that expression of the gene Bmi-1, plays an important role in self-renewal divisions, but not haemopoietic differentiation, by regulating the expression of genes involved in cell fate decisions, as well as cell survival, anti-proliferation and stem cell-associated genes (Park *et al.* 2003). Bmi-1 is a member of the Polycomb group (PcG) family that is linked to malignant transformation and lymphocyte development, and expression of this gene declines during haemopoietic development (van der Lugt *et al.* 1994; van der Lugt *et al.* 1996; Kiyono *et al.* 1998; Lessard *et al.* 1998; Akashi *et al.* 2003).

While the factors governing the decision of a stem cell to differentiate or self-renew are poorly understood, two models have been proposed. These are termed the stochastic and deterministic models.

1.4.1 The Stochastic Model of Stem Cell Decision

The stochastic model of stem cell function was first proposed in 1964 by Till *et al.* using computer simulation and experimental data, and since then has been supported by further research (Ogawa 1993). This stochastic model predicts that the likelihood that a stem cell will self-renew is an intrinsic property of the progenitor cell and is limited by probability

constant, *p*, *p* estimated to be between 0.55-0.60 (Till *et al.* 1964) or a value of 0.5 (Ogawa 1993). If *p* was to fall below a value of 0.5, then the stem cell pool would be exhausted.

The greatest criticism of the stochastic model has been the use of methylcellulose culture to demonstrate reconstitution potential of HSC. Because physiological self-renewal cannot be observed in methylcellulose culture it is very difficult to draw parallels with what is occurring *in vivo* (Ogawa 1993). However the idea that intrinsic factors, such as transcription factor expression, may specify the individual fate of stem cells has been backed by various research groups to date. For example, Mayani *et al.* (1993) examined HSC proliferation and differentiation in serum-free culture systems supplemented with different cytokine combinations, and found that asymmetric divisions of cells in early phases of haemopoiesis could not be influenced by the addition of specific cytokines. Furthermore, the relative proportions of granulocyte/macrophage, erythroid and multipotent progenitors remained consistent regardless of the cytokine combination used (Mayani *et al.* 1993).

1.4.2 The Deterministic Model of Stem Cell Decision

The deterministic model of HSC differentiation proposes that the decision of a stem cell to differentiate or self-renew can be manipulated by external factors such as cytokines, or predicted on the basis of lineage marker expression (Morrison and Weissman 1994). In *in vivo* terms, this model suggests that the stromal microenvironment is the determining factor with regard to whether HSC self-renew or differentiate. Data in support of the deterministic model comes from the observation that heterogeneity exists with regard to the level of Mac-1 and CD4 expression on highly enriched HSC (Morrison and Weissman 1994). Long term reconstitution experiments with HSC populations isolated on the basis of Mac-1 and CD4 expression revealed that each population displayed distinct self-renewal potentials. Only cells negative for Mac-1 and CD4 were enriched for cells capable of self-renewal, whereas the majority of cells expressing low levels of CD4 and Mac-1 failed to self-renew or show reconstitution (Morrison and Weissman 1994). The authors argued that since self-renewal could be predicted on lineage marker expression then self-renewal and therefore reconstitution potential must be deterministic (Morrison and Weissman 1994). This hypothesis can be extended to the stem cell pool where HSC exist in a limited

number of differentiation states rather than as a continuum of self-renewal potentials (Morrison *et al.* 1995).

1.4.3 Stochastic & Deterministic Control of Stem Cell Fate

A combination of both the deterministic and stochastic models may govern HSC self-renewal as results may be interpreted in a way that supports both models. For example, HSC heterogeneity has been observed at both the level of cytokine receptor expression and in the number of cells expressing each type of receptor (for review, see Enver *et al.* 1998). The heterogeneity observed in this population of cells is consistent with the random, stochastic model while heterogeneity, with respect to receptor coexpression, would enable the cells to receive instructive signals from a range of cytokines and support the deterministic model (Enver *et al.* 1998).

If the self-renewal and differentiation of HSC was solely controlled by stochastic mechanisms, then it would be extremely difficult for stem cell homeostasis to be maintained in the event of an *in vivo* disturbance, such as blood loss, where rapid differentiation towards a particular cell lineage is required. It has therefore been hypothesized that primitive cell differentiation is under stochastic control, but once a cell becomes more differentiated and lineage restricted, deterministic processes become more important and allow rapid responses to environmental changes (Enver *et al.* 1998).

1.5 Haemopoietic Stem Cell Differentiation

The first stage of HSC differentiation is a loss of the cell's ability to self-renew. This stage is followed by commitment to either the lymphoid or myeloid lineages, and the sequential commitment to more restricted phenotypes until they form functionally distinct mature blood cells (Cantor and Orkin 2001).

The differentiation of stem cells requires both exit from the uncommitted state and entry into a particular developmental pathway. Data from *Caenorhabditis elegans* suggests that these two aspects may be controlled independently, as exit from the stem cell state requires both the loss of a protein that normally inhibits the expression of genes involved in commitment to a particular lineage and the presence of positive transcriptional regulators

(reviewed in Morrison *et al.* 1997). Whether these two aspects are also independently controlled in mammals is still unknown.

The stochastic and deterministic model can also be applied to cells once they begin to differentiate, these being termed the "selective" and "instructive" models. In the selective model, whether stem cells commit to a particular lineage is independent of growth factors, the growth factors acting only to control the survival or proliferation of committed progenitors (Mayani *et al.* 1993). Alternatively, in the instructive model, growth factors determine what lineage a stem cell will differentiate towards, rather than supporting survival of lineage committed progenitors (Morrison *et al.* 1997).

Exit from the stem cell state is followed by transition to common lymphoid progenitors or common myeloid progenitors. Common lymphoid progenitors then give rise to B and T cells and natural killer cells (Kondo *et al.* 1997). Common myeloid progenitors differentiate into granulocytes, monocytes/macrophages, megakaryocytes or erythrocytes, with granulocytes being subdivided into neutrophils, eosinophils or basophils (Akashi *et al.* 2000).

1.6 Regulation of Haemopoietic Stem Cell Differentiation

Current models of haemopoietic differentiation suggest that lineage commitment is determined by extrinsic factors such as adhesion molecules, the bone marrow stroma and cytokines, intrinsic factors such as transcription factors, or a combination of extrinsic and intrinsic mechanisms (reviewed in Tenen *et al.* 1997). It has been proposed that intrinsic factors play a major role in determining lineage commitment of HSC, while extrinsic mechanisms, such as cytokines, support the survival of committed cells (Tenen *et al.* 1997). There is increasing evidence, however, that growth factors, cytokines and chemokines secreted by haemopoietic cells are involved in intercellular cross talk networks, thereby regulating haemopoiesis (Janowska-Wieczorek *et al.* 2001).

1.6.1 Extrinsic Regulation of Haemopoietic Differentiation

Growth factors, cytokines and chemokines are secreted in the bone marrow microenvironment by accessory cells such as fibroblasts, macrophages, endothelial cells

and osteoblasts, by T cells, and by haemopoietic cells themselves (Janowska-Wieczorek *et al.* 2001; Majka *et al.* 2001). While cytokines may influence the survival and proliferation of committed progenitor cells, there is no convincing evidence that suggests cytokines can instruct the differentiation of HSC (Ogawa 1993).

The detection of growth factor, cytokine and chemokine secretion by early and differentiated haemopoietic cells provides evidence that these factors may form the basis of intercellular cross-talk networks and be involved in regulating haemopoiesis in an autocrine/paracrine fashion (Majka *et al.* 2001). These cytokines include stem cell factor (SCF, KL), Flt-3 ligand, thrombopoietin (TPO), transforming growth factor-β1 (TGF-β1), TGF-β2 and interleukin-8 (IL-8); neither IL-3 nor granulocyte colony stimulating factor (G-CSF) have been detected (Majka *et al.* 2001). Human bone marrow-derived $CD34^+$ cells have also been found to express mRNA for growth factors that play a role in stimulating cell proliferation (such as TPO and IL-1), growth factors that have an inhibitory role on cell proliferation (such as tumour necrosis factor-α (TNF-α) and interferon-α (IFN-α)), and growth factors that protect the cells from undergoing apoptosis (such as SCF, Flt-3 and TPO) (Janowska-Wieczorek *et al.* 2001; Majka *et al.* 2001). These endogenously secreted factors may regulate cellular homing by stimulating the secretion of other regulatory molecules and by modulating the expression of adhesion molecules.

Semisolid culture assays have been used to examine the effect of growth factors on haemopoiesis, with the advantage of this assay over liquid cultures being that the progeny of individual precursor cells remain physically localized. The interesting conclusion from these experiments was that, while no two regulators stimulated exactly the same pattern of colony formation based on colony numbers and phenotypes, more than one factor could stimulate the formation of exactly the same type of colony (for review, see Metcalf 1993). For example, neutrophilic colony formation could be stimulated with G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, IL-6 or SCF, and likewise, several growth factors could stimulate megakaryocyte, eosinophil and mast cell development. Such results suggest redundancy within the cytokine system, and it has been suggested that restriction in the expression of growth factor receptors to particular subsets of cells ensures cytokine specificity (Metcalf 1993).

1.6.1.1 Role of Tyrosine Kinases in Haemopoiesis

The regulation of haemopoiesis by growth factors, cytokines and chemokines is mediated through binding to high-affinity receptors. Three families of cell surface receptors can be characterised on the basis of their mode of action, including enzyme-linked receptors, ion channel-linked receptors, and G-protein-linked receptors. It is only the enzyme-linked receptors that are involved in growth factor and cytokine signalling, acting directly as enzymes, or in association with enzymes, to activate intracellular signalling cascades (reviewed in Ravandi *et al.* 2002).

There are currently five classes of enzyme-linked receptors: receptor guanylyl cyclases, receptor tyrosine phosphatases, receptor serine/threonine kinases, receptor tyrosine kinases (RTKs), and tyrosine kinase-associated receptors (Ravandi *et al.* 2002). The majority of cytokines and growth factors signal through cell surface receptor tyrosine kinases or tyrosine kinase-associated receptors, with the human genome containing approximately 90 genes that encode for tyrosine kinases, 58 of which are RTKs (Ravandi *et al.* 2002; Reilly 2002).

RTKs are membrane-bound enzymes composed of an extracellular ligand-binding domain, a transmembrane domain, and a highly conserved intracellular domain that is involved in phosphorylation of tyrosine residues (reviewed in Ravandi *et al.* 2002). They mediate cellular responses to a wide range of extracellular signals that are involved in regulating cell survival, proliferation, migration and differentiation (reviewed in Scheijen and Griffin 2002). Binding of ligand to RTKs initiates a cascade of events that include receptor homodimerisation, activation of intrinsic kinase activity, intermolecular tyrosine trans-phosphorylation and phosphorylation of substrates (Scheijen and Griffin 2002). Subdivision of RTKs into 4 classes can be made on the basis of their structure: RTK class I, such as receptors for epidermal growth factor; RTK class II, such as receptors for insulin-like growth factor; RTK class III, such as receptors for platelet derived growth factor (PDGFR), FMS-like tyrosine kinase-3 (Flt-3-R), macrophage colony stimulating factor (c-fms) and stem cell factor (c-kit)); and RTK class IV, such as receptors for fibroblast growth factor (reviewed in Ravandi *et al.* 2002).

The class III RTK receptors and their ligands play an important role in regulating the survival, proliferation and differentiation of HSC. It is therefore not surprising that in several cancers such as leukaemia, over expression and/or constitutive activation of RTKs is observed. For example, inappropriate activation of the Ras signalling proteins that are involved in relaying signals from cell surface receptors have been described in human leukaemias (Janssen *et al.* 1987).

1.6.1.2 Jak-STAT Signalling

The Janus kinase (Jak) family of cytoplasmic tyrosine kinases plays a pivotal role in signal transduction via cytokine receptors, and is currently comprised of four family members (Jak1, Jak2, Jak3 and tyk2) (Ihle and Kerr 1995). Jaks become phosphorylated following ligand binding, and these activated Jaks subsequently phosphorylate a number of substrates including cytokine receptors; the phosphorylated cytokine receptors then provide a docking site for STATs (signal transducers and activators of transcription) and other intracellular signalling molecules (Ihle and Kerr 1995). STATs are in turn phosphorylated by activated Jaks, after which STATs either homodimerize or heterodimerize and translocate to the nucleus (Ihle 1996). It is now accepted that this process serves as a high-speed connection between numerous cytokine receptors and their downstream biological responses (Liu *et al.* 1998).

The existence of 4 different Jaks and at least 7 different STATs, all with different DNA binding and transactivation potentials, enables specificity in the Jak-STAT cytokine signalling pathway and is a critical means by which the response of haemopoietic cells to a wide range of cytokines is mediated (Liu *et al.* 1998; de Groot *et al.* 1999). In addition, cell type-specific differences in STAT expression are likely to play a role in determining the specific response of cells to cytokines over the various lineages and at different stages of maturation (Ihle and Kerr 1995).

1.6.1.3 Growth Factors & Cytokines Involved in Haemopoiesis

1.6.1.3.1 Erythropoietin

Erythropoietin (EPO) is a restricted haemopoietic regulator that increases the number of developing erythroid precursors from marrow and has a dominant action on mature

erythroid precursors (for review, see Spivak 1986). The use of recombinant EPO in colony assays has demonstrated that this growth factor is an effective stimulus for erythroid colony formation but it had no effect on megakaryocytic colony formation, megakaryocytic differentiation or the formation of other colony types (Koike *et al.* 1986; Ganser *et al.* 1988).

1.6.1.3.2 Flt-3 Ligand

The Flt-3 receptor is expressed on primitive haemopoietic cells and its ligand, Flt-3 ligand (Flt-3l), exerts stimulative effects on HSC (Banu *et al.* 1999). Flt-3l is unable to support colony growth from $CD34^+$ cells alone, however in combination with cytokines such as IL-3, IL-6, G-CSF, GM-CSF and SCF, it significantly augments the growth of myeloid colonies (Brashem-Stein *et al.* 1996; Banu *et al.* 1999). Flt-3l has also been implicated in the expansion and mobilisation of haemopoietic progenitor cells (Robinson *et al.* 2000).

1.6.1.3.3 Granulocyte Colony Stimulating Factor

G-CSF is the principal growth factor that regulates granulopoiesis (Richards *et al.* 2003). The use of purified G-CSF in colony assays has been shown to stimulate the formation of granulocytic colonies and, at high concentrations of G-CSF, low levels of macrophage and granulocyte-macrophage colony formation are observed (Metcalf and Nicola 1983). While G-CSF is able to promote the survival and/or proliferation of some multipotential, erythroid, and eosinophil progenitors in foetal liver this growth factor is unable to sustain the proliferation of these cells and induce colony formation (Metcalf and Nicola 1983).

The biologic effects of G-CSF are mediated through the G-CSF receptor (G-CSF R), a member of the haemopoietic class I cytokine receptor family that is expressed on multipotential haemopoietic progenitor cells, and more differentiated cells of the myeloid lineage (Demetri and Griffin 1991; McKinstry *et al.* 1997; Miyamoto *et al.* 2002).

1.6.1.3.4 Granulocyte-Macrophage Colony Stimulating factor, Interleukin-3 & Interleukin-5

GM-CSF, IL-3 and IL-5 are related haemopoietic cytokines that are members of the short-chain 4-α-helical bundle subset of cytokines (reviewed in Martinez-Moczygemba and

Huston 2003). These cytokines act synergistically to promote the differentiation of myeloid cells and play an important role in protective immunity (Martinez-Moczygemba and Huston 2003).

GM-CSF is produced by activated T lymphocytes, eosinophils, mast cells, basophils, macrophages, endothelial cells and bone marrow stromal cells (Martinez-Moczygemba and Huston 2003). It affects the survival, growth and differentiation of numerous haemopoietic lineages, including the formation of granulocytes and mixed granulocyte-macrophage colonies in soft agar, and the differentiation of monocytes into macrophages.

In contrast to growth factors that affect a specific subset of cells such as EPO, IL-3 affects stem cells, erythroid cells, granulocytes, macrophages, eosinophils, megakaryocytes, and mast cell and B cell precursors (Metcalf *et al.* 1987). IL-3 promotes the development of HSC following activation through the IL-3 receptor complex, and is thought to play a role in promoting the survival of more differentiated HSC (Brandt *et al.* 1994). In particular, IL-3 appears to support the proliferation of multipotential progenitors once they exit from G_0, but does not maintain the terminal stages of haemopoiesis alone (Ogawa 1993). As haemopoietic progenitor cells differentiate towards the neutrophil lineage the cells lose responsiveness to IL-3, suggesting that the effects of IL-3 on haemopoiesis are restricted to the intermediate stages of haemopoiesis (Lopez *et al.* 1988; Ogawa 1993).

Significant levels of IL-3 and GM-CSF have not been observed in the bone marrow microenvironment, and it was initially believed that the main function of these cytokines was to stimulate haemopoiesis in cases of emergency (Hara and Miyajima 1996). The generation of knockout mice lacking functional IL-3, and GM-CSF failed to reveal any major haematological defect other than a reduced number of eosinophils, and haemopoietic recovery after cytotoxic drug treatment was normal (Nishinakamura *et al.* 1996).

IL-5 is a cytokine that is mainly secreted by T helper cells, although it is also produced by activated mast cells and eosinophils (Martinez-Moczygemba and Huston 2003). The main cellular targets of IL-5 are eosinophils and eosinophilic progenitor cells, with stimulation of eosinophils with IL-5 ultimately inhibiting their apoptosis (Yamaguchi *et al.* 1991).

The biological functions of GM-CSF, IL-3 and IL-5 are mediated by high-affinity membrane-bound receptors that are composed of a common β receptor signal transducing subunit (βc) and a specific ligand-binding subunit (α) (Hayashida *et al.* 1990; Kitamura *et al.* 1991; Tavernier *et al.* 1991). While the βc subunit is common for each of these cytokines, the α receptor subunit is unique to each growth factor and only binds to its specific ligand (Hayashida *et al.* 1990; Kitamura *et al.* 1991; Tavernier *et al.* 1991; Rozwarski *et al.* 1994; Martinez-Moczygemba and Huston 2003).

The GM-CSFRα is expressed on all myeloid lineages and $CD34^+$ progenitor cells, while IL-5Rα expression is seen on $CD34^+$ progenitor cells and eosinophils (Sehmi *et al.* 1997; Martinez-Moczygemba and Huston 2003). IL-3Rα expression correlates with the types of cells that are responsive to IL-3 and includes $CD34^+$ progenitor cells, basophils, mast cells, and dendritic cells.

Although the GM-CSF, IL-3 and IL-5 receptors do not have intrinsic tyrosine kinase activity, ligand binding induces tyrosine phosphorylation of the receptor within the cytoplasmic domain of the β chain, and signal transduction pathways such as Ras are activated (Itoh *et al.* 1996b; Guidez *et al.* 1998). Other signal transduction pathways activated include the Jak/STAT pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphotidylinositol3-kinase (PI3-K) pathway (Brizzi *et al.* 1994; Guthridge *et al.* 1998; Scott and Begley 1999; Woodcock *et al.* 1999).

1.6.1.3.5 Interleukin-6

IL-6 exhibits a range of biological activities including effects on haemopoiesis. IL-6 supports the proliferation of granulocyte and macrophage progenitors and, acting synergistically with IL-3, it augments the expansion of primitive haemopoietic precursor cells in blast cell colony assays (Ikebuchi *et al.* 1987). This latter effect has, at least in part, been attributed to a decrease in the G_0 phase of the cell cycle (Ikebuchi *et al.* 1987).

IL-6 augments megakaryocytic, erythroid and myeloid colony growth in the presence of macrophage colony stimulating factor (M-CSF), GM-CSF and IL-6 (Rennick *et al.* 1989).

This suggests that IL-6 interacts with a range of growth factors to regulate the growth of progenitor cells at different stages of lineage commitment and maturation (Rennick *et al.* 1989).

1.6.1.3.6 Macrophage Colony Stimulating Factor & c-fms

M-CSF (also known as colony-stimulating factor-1, CSF-1) is a macrophage-lineage specific growth factor that supports the proliferation, differentiation and survival of cells from the mononuclear phagocyte lineage (reviewed in Sherr 1990). It is a 70-90kDa homodimeric glycoprotein (Stanley and Heard 1977) that is primarily synthesized by fibroblasts, however its production can also be induced following stimulation of endothelial cells, monocytes and macrophages with other growth factors, such as IL-3, IL-4 and GM-CSF (Seelentag *et al.* 1987; Sherr 1990).

M-CSF has a direct effect on the proliferation of late monocytic and earlier granulomonocytic committed progenitors and also acts indirectly on haemopoiesis by stimulating production of various cytokines such as G-CSF and IFN, tumour necrosis factor and IL-1 by macrophages (reviewed in Sherr 1990). Whether M-CSF also plays a role in the proliferation and differentiation of primitive progenitors is controversial (Fixe and Praloran 1998).

The biological effects of M-CSF are mediated by a unique receptor, c-fms (M-CSFR, CSF-1R), which is encoded by a protooncogene that is the cellular counterpart of the *v-fms* oncogene encoded by the Susan McDonough and Hardy-Zuckerman strains of the feline sarcoma virus (Sherr *et al.* 1985). The receptor c-fms is a member of the type III receptor tyrosine kinase (RTK) family, which also includes c-kit (Yarden *et al.* 1987), Flt-3 (Rosnet *et al.* 1993), PDGFRα (Claesson-Welsh *et al.* 1989) and PDGFRβ (Yarden *et al.* 1986).

Unlike many growth factor receptors that have multiple ligands, M-CSF is the single known ligand for c-fms (Bourette and Rohrschneider 2000). Upon M-CSF binding to the extracellular domain of c-fms the receptor is induced to dimerize and then trans-autophosphorylate several cytoplasmic tyrosine residues. Only seven of the twenty tyrosine residues in the cytoplasmic domain have been reported to be phosphorylated

following ligand binding and, once phosphorylated, subsequently interact with primary adapter proteins which then initiate signalling along specific pathways (Bourette and Rohrschneider 2000). The best characterised pathway is activation of Ras and subsequently MAP kinase (MAPK), which translocates to the nucleus and phosphorylates Elk 1 transcription factor (Bourette and Rohrschneider 2000). A second major signalling pathway initiated by M-CSF is binding of p85-PI3K (phosphoinositide 3-kinase) to activated forms of c-fms, with the PI3K protein being part of the signalling cascade of most growth factors (Bourette and Rohrschneider 2000).

The c-fms gene is expressed at low levels in monocytes and is markedly upregulated during the differentiation of monocytes into macrophages. In the absence of M-CSF the mature cell-surface form of c-fms is relatively stable and has a half-life of three to four hours (Rettenmier *et al.* 1987). Upon ligand binding, however, receptor expression is downregulated by internalisation of the ligand-receptor complex and degradation within lysosomes (Rettenmier *et al.* 1987). At saturating concentrations of M-CSF complete receptor degradation is seen within 15 minutes, such that the cells are non-responsive to M-CSF stimulation until new ligand is synthesized and re-expressed at the cell surface (Sherr 1990).

1.6.1.3.7 Platelet Derived Growth Factor

Platelet derived growth factor (PDGF) acts as a potent stimulant for the proliferation of mesenchymal cells and plays a vital role in physiological wound repair and the pathogenesis of a number of proliferative diseases (reviewed in Rosenkranz and Kazlauskas 1999). It is produced by a range of cell types including fibroblasts, keratinocytes, neurons, macrophages and platelets (reviewed in Heldin and Westermark 1999), and exists as a homo and heterodimeric protein that is composed of homologous A and B chains. All 3 isoforms of PDGF (AA, AB, BB) exhibit biological activity and it is thought that assembly of these dimers is a random process (Claesson-Welsh *et al.* 1989; Heldin and Westermark 1999; Rosenkranz and Kazlauskas 1999).

Within the haemopoietic system, PDGF promotes the growth of multilineage haemopoietic precursor cells, long-term culture-initiating cells and erythropoietic cells (Dainiak *et al.*

1983; Yan *et al.* 1993; Su *et al.* 2002). PDGF-BB isoforms have been shown to stimulate murine colony-forming unit-granulocyte-monocyte (CFU-GM) proliferation from bone marrow cells in a dose-dependent manner, and this growth was inhibited by the addition of anti-GM-CSF, anti-IL-3 and anti-IL-6 antibodies (Yang *et al.* 2001). Since stimulation of purified $CD34^+$ cells with PDGF alone had no effect on the growth of GFU-GM, these results suggest that PDGF mediates its effects on haemopoiesis through inducing endogenous production of GM-CSF, IL-3 and IL-6 by bone marrow stromal cells (Yang *et al.* 2001).

A growth promoting effect of PDGF on $CD34^+$ cells, when used in combination with other stimulatory cytokines such as TPO, IL-3 and IL-6, has also been proposed to result from secondary effects on accessory cells, rather than PDGF acting directly on $CD34^+$ cells (Su *et al.* 2002). This hypothesis is supported by the observation that PDGF receptor expression is not detectable on freshly isolated $CD34^+$ cells (Su *et al.* 2002).

The biological response to PDGF is mediated by the platelet derived growth factor receptor (PDGFR), which is a member of the type III receptor tyrosine kinase family (Yarden *et al.* 1986; Claesson-Welsh *et al.* 1989). This receptor, therefore, shows structural similarities to c-fms and c-kit receptor tyrosine kinases (Yarden *et al.* 1986; Yarden *et al.* 1987). Two forms of PDGFR exist, and are structurally related: the type α PDGFR which binds to all 3 isoforms of PDGF, and the type β PDGFR which binds to PDGF-BB and PDGF-AB isoforms. This suggests that these receptors have distinct biological roles and transduce overlapping, but not identical, signals (Heldin and Westermark 1999; Rosenkranz and Kazlauskas 1999).

As a result of the differences in the binding specificity of PDGF isoforms, cell responses are dependent on which two receptors the cells express. For example, cells that are main targets of PDGF, such as fibroblasts and smooth muscle cells, express both α and β receptors while other cell types such as platelets and endothelial cells express either α or β receptors (reviewed in Heldin and Westermark 1999). Following ligand binding PDGFR subunits dimerise and autophosphorylate on tyrosine residues, activating a variety of

molecules involved in signal transduction pathways such as Src family tyrosine kinases, PI3-kinases and phospholipases (reviewed in Rosenkranz and Kazlauskas 1999).

1.6.1.3.8 Stem Cell Factor & c-kit

SCF (also known as kit ligand, mast cell growth factor, or steel factor) is a haemopoietic cytokine produced by the bone marrow stroma that binds to the receptor c-kit and, in combination with other cytokines, promotes the growth of all haemopoietic lineages (for review, see Broudy 1997). The absence of SCF or c-kit in knockout mice results in death *in utero* due to severe macrocytic anaemia and, in addition to the importance of SCF in development, this cytokine is also required throughout adult life for maintaining normal haemopoiesis (Broudy 1997).

SCF has been implicated in promoting the survival of HSC *in vitro* (Brandt *et al.* 1994), and has been shown to accelerate the entry of enriched populations of HSC into the cell cycle. It does not appear to play a role in HSC self-renewal (Gore *et al.* 1995; Broudy 1997). Although SCF does not support significant colony growth alone it synergises with other cytokines, such as EPO, IL-3, GM-CSF and G-CSF, to increase the size and number of erythroid, macrophage and megakaryocytic colonies (McNiece *et al.* 1991).

The receptor, c-kit, is a tyrosine kinase closely related to the receptors for platelet derived growth factor and macrophage colony stimulating factor and is rapidly induced to dimerize following interaction with its ligand. This is followed by an increase in autophosphorylation activity and activation of multiple downstream signal transduction components, including phosphotidylinositol-3-kinase (PI3 kinase), Src family members, the JAK-STAT pathway and the Ras-Raf-MAP kinase cascade (Linnekin 1999). The expression of c-kit mRNA has been detected in mast cells, melanocytes, megakaryocytes and the bone marrow (including 70% of $CD34^+$ cells), as well as in non-haemopoietic cell types (Ashman 1999).

1.6.1.3.9 Thrombopoietin & c-Mpl

TPO is the primary regulator of megakaryocyte differentiation, supporting the proliferation, differentiation and maturation of megakaryocytes and their precursors, and

inducing their fragmentation into platelets (Kaushansky 1995). In semi-solid culture assays, TPO stimulates megakaryocyte proliferation, an effect that is augmented by IL-3 and SCF (Broudy *et al.* 1995). Mice lacking either TPO or its receptor, c-Mpl, are viable but display abnormal megakaryocyte and platelet development, and are deficient in haemopoietic progenitor cell lineages and the ability to regulate HSC production (Kimura *et al.* 1998). Megakaryocytes and platelets produced in the absence of TPO or c-Mpl, however, appear to be morphologically and functionally normal, suggesting that TPO is involved in maintaining megakaryocyte numbers rather than in controlling their maturation (Murone *et al.* 1998). Other cytokines that are able to induce megakaryocyte expansion include IL-1, IL-11, SCF, EPO, IL-3, IL-6 and GM-CSF, however their contribution is easily surpassed by the effect of TPO (Murone *et al.* 1998).

In addition to its role in megakaryopoiesis, TPO is involved in supporting the survival of HSC (Borge *et al.* 1997). When HSC were cultured in the presence of TPO alone, TPO supported cell survival but failed to induce proliferation or colony growth (Sitnicka *et al.* 1996; Itoh *et al.* 1996a). In combination with IL-3 and SCF, however, TPO had several synergistic effects such as shortening cell cycling time and increasing cloning efficiency (Sitnicka *et al.* 1996; Itoh *et al.* 1996a).

1.6.1.4 Cytokine Synergism

Colony formation assays using $CD34^+$ cells have revealed that IL-3 or GM-CSF are required for normal primitive haemopoietic progenitor cells to survive in G_0, even when these progenitor cells are dormant (Leary *et al.* 1992). The progenitor cells remain in G_0 until they are triggered into active cycling by IL-3 in combination with a synergistic factor such as IL-6, IL-11, SCF or G-CSF (Leary *et al.* 1992). This confirms previous observations where, in contrast to primitive myeloerythroid progenitor cells, HSC require multiple factor signalling to be activated and subsequently initiate colony formation (Heimfeld *et al.* 1991).

IL-3, IL-6 and GM-CSF act on multiple haemopoietic lineages, while IL-5, G-CSF, EPO and TPO stimulate more restricted lineages of haemopoietic cells (Hara and Miyajima 1996). IL-3, GM-CSF and IL-5 are not expressed in bone marrow stroma and the

generation of mice deficient in each of these cytokines or their receptors has suggested that these factors are dispensable for haemopoiesis (Nishinakamura *et al.* 1996). Such results also support the functional overlap of various cytokines.

Large volume suspension cultures of $CD34^+$ cells have been used to determine the most potent combination of cytokines that promote cellular differentiation along the myeloid lineage (Haylock *et al.* 1992). A combination of IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF was identified to be the most effective, with a 1324-fold increase in nucleated cells (predominantly neutrophilic) occurring at day fourteen. Stimulation with each of these cytokines alone yielded a poor increase in cell number (Haylock *et al.* 1992).

Although specific combinations of cytokines that support the production of myeloid (Haylock *et al.* 1992), megakaryocytic (Kaushansky 1995), erythroid, dendritic cells and activated lymphocytes from HSC have been identified, researchers are still unable to expand HSC capable of self-renewal divisions. While past interest has focussed on identifying growth factors that promote maturation and differentiation, the enormous therapeutic potential of HSC has shifted research towards identifying the factors that promote self-renewal. Multiple self-renewal divisions of stem cells has been observed in HSC cultured in the presence of Flt-3 ligand, SCF, IL-3, IL-6, and G-CSF (Glimm and Eaves 1999), however large (>10 fold) and continuing expansion of cells with stem cell activity has still not been achieved. This is partly attributable to the cells undergoing apoptosis (Williams *et al.* 1990), however simply blocking apoptosis in the absence of correct stimuli does not permit successful culture of self-renewing HSC, as the cells undergo cellular differentiation (Domen and Weissman 1999).

1.6.2 Intrinsic Regulation of Haemopoietic Differentiation

1.6.2.1 Transcriptional Regulation of Haemopoietic Stem Cell Differentiation

Transcription factors are regulatory proteins that bind to promoter DNA sequences and exert a range of functions that include the folding of DNA into distinct domains, initiation of DNA replication and control of gene transcription (Barreda and Belosevic 2001). The 3 dimensional structure of the DNA-binding motifs is typically used for classifying transcription factors, and the majority of these transcription factors are characterised by

zinc finger, helix-turn-helix, leucine zipper and winged helix motifs (Barreda and Belosevic 2001).

Recent evidence suggests that variation in the expression of specific combinations of transcription factors plays a crucial role in determining whether a cell will commit, proliferate, differentiate or undergo apoptosis (Hu *et al.* 1997). The classical model of haemopoietic lineage commitment dictates that lineage-specific transcription factors act in a positive manner, driving the cell towards a particular lineage. It is now apparent that the molecular mechanisms of cellular differentiation are more complex and that positively acting transcription factors may simultaneously exert inhibitory effects on alternate lineage gene programs by directly suppressing the action of opposing transcription factors (Cantor and Orkin 2001). Following an increase in the expression of one lineage specific transcription factor relative to another (Cantor and Orkin 2001), and as a result of interactions between these transcription factors, co-regulatory molecules and specific DNA binding sequences (Barreda and Belosevic 2001), the phenotype of the cell is determined.

A summary of various transcription factors that play a vital role in the differentiation decisions of haemopoietic cells is displayed in Table 1.1. These include PU.1, which is involved in granulocytic/monocytic differentiation (Voso *et al.* 1994); GATA-1, which is involved in erythroid and megakaryocytic lineage development (Shivdasani *et al.* 1997; Weiss *et al.* 1997); NF-E2 (nuclear factor- erythroid 2) which is thought to be a major enhancer for globin gene expression in maturing erythroid cells (Ney *et al.* 1990a; Ney *et al.* 1990b; Andrews *et al.* 1993a); and c-Myb which plays an important role in the differentiation of macrophages and lymphocytes (Allen *et al.* 1999). In addition, Figure 1.1 clarifies the stage of differentiation at which these particular transcription factors are most important.

Table 1.1. Transcription Factors Involved in the Regulation of Haemopoiesis.

Transcription Factor	Family	DNA Binding Domain	Expression	Putative Function	References
PU.1	ets	Winged helix-loop-helix	Exclusive to haemopoietic cells. High levels: •Monocytes •B cells •Granulocytes •Megakaryocytes •Mast cells •Immature erythroid cells Low levels: •Mature erythrocytes	Regulates expression of lymphoid and myeloid genes such as: •Immunoglobulin heavy chain gene in B cells. •G-CSF receptor, GM-CSF receptor, CD11b and CD18 in myeloid cells. •↓ myeloid differentiation •↑ erythroid differentiation	Klemsz *et al.* 1990; Rosmarin *et al.* 1992; Nelsen *et al.* 1993; Pahl *et al.* 1993; Chen *et al.* 1995; Hohaus *et al.* 1995; Smith *et al.* 1996; DeKoter and Singh 2000
GATA-1	GATA	(A/T)GATA(A/G)	High levels: •Erythroid cells •Megakaryocytes •Eosinophils •Mast cells Low levels: •Multipotential progenitor cells	•Coordinates the transition from proliferation to differentiation •Involved in erythroid differentiation. • Involved in late megakaryocytic differentiation.	Shivdasani *et al.* 1997; Weiss *et al.* 1997
GATA-2	GATA	(A/T)GATA(A/G)	•Early haemopoietic progenitor cells •Mast cells •Megakaryocytes	•Essential during early stages of haemopoiesis. •Involved in terminal differentiation of mast cells. • ↓ erythroid differentiation •↑ megakaryocytic differentiation	Tsai *et al.* 1994; Tsai and Orkin 1997; Ikonomi *et al.* 2000
NF-E2 (nuclear factor-erythroid 2)	Basic-leucine zipper superfamily	Extended AP-1 motif (T/C)TGCTGA(C/G)TCA(T/C)	•Erythroid cells •Megakaryocytic cells •Mast cells	•Major enhancer for globin gene expression in maturing erythroid cells. •Co-expressed with GATA-1.	Ney *et al.* 1990a; Andrews *et al.* 1993a; Andrews *et al.* 1993b

Table 1.1 (continued). Transcription Factors Involved in the Regulation of Haemopoiesis.

Transcription Factor	Family	DNA Binding Domain	Expression	Putative Function	References
SCL (stem cell leukaemia)	Helix-loop-helix	Ebox motifs	•Erythroid cells •Mast cells •Early myeloid cells	•Undergoes coordinated biphasic modulation with GATA-1 during erythroid and myeloid differentiation. •↓ myeloid differentiation •↑ erythroid differentiation	Begley *et al.* 1989; Bernard *et al.* 1990; Chen *et al.* 1990; Visvader *et al.* 1991; Green *et al.* 1992; Ellenberger *et al.* 1994
c-Myb		Three homologous tandem repeats of 52 amino acids.	•Haemopoietic progenitor cells	•Protooncogene that is a cellular homologue of the v-Myb gene. •Plays a role in the differentiation of macrophages and lymphocytes. •Involved in early T cell development.	Klempnauer *et al.* 1982; Mucenski *et al.* 1991; Tanikawa *et al.* 1993; Allen *et al.* 1999
C/EBP	Basic-leucine zipper superfamily	CAAT	• Restricted to myeloid cells within the haemopoietic system	•Implicated in monocyte development. • Six family members.	Scott *et al.* 1992; Lekstrom-Himes 2001; Jones *et al.* 2002

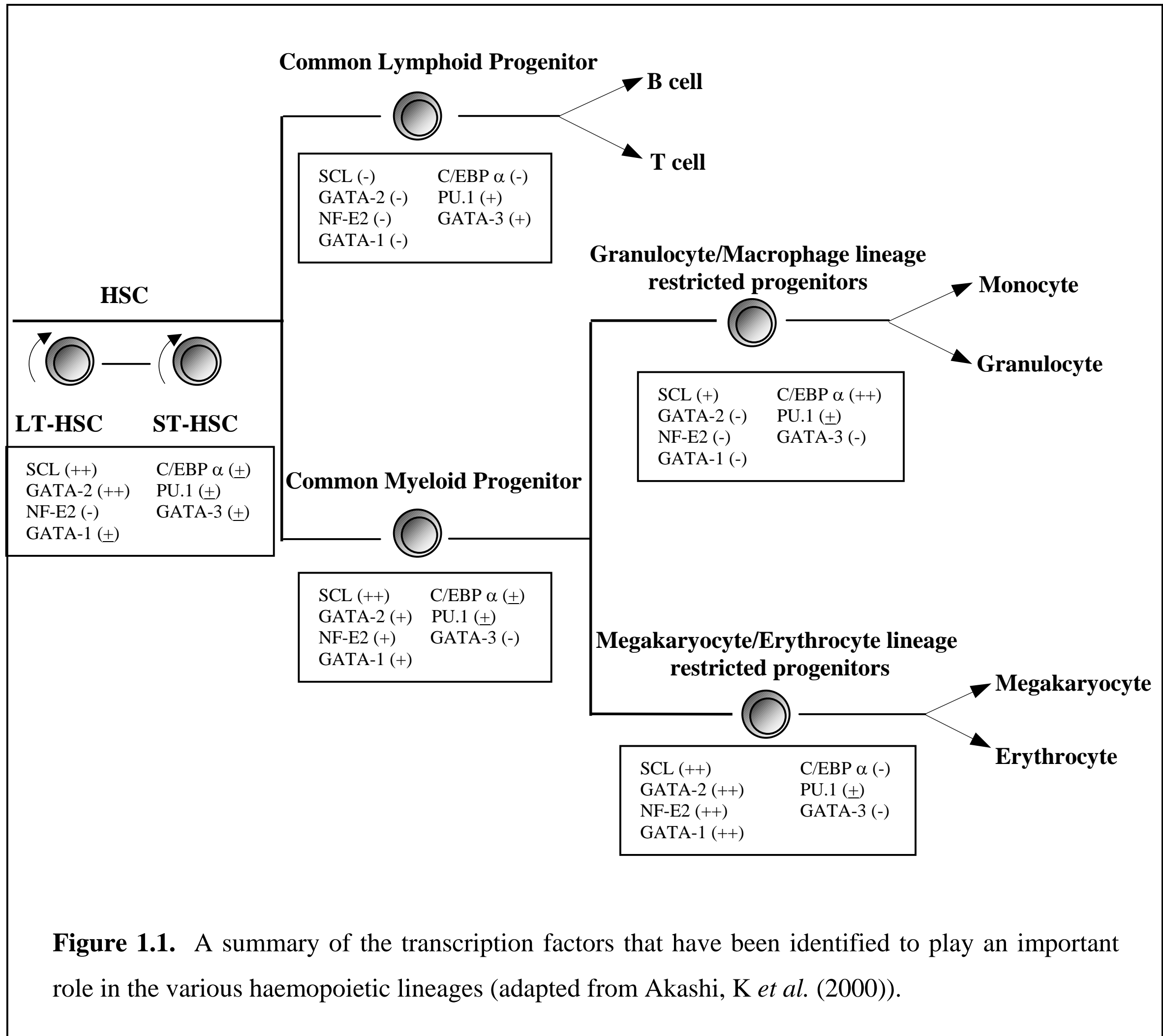


Figure 1.1. A summary of the transcription factors that have been identified to play an important role in the various haemopoietic lineages (adapted from Akashi, K *et al.* (2000)).

1.6.2.1.1 Regulation of Transcription Factor Activity

The expression of lineage specific transcription factors overlaps in early multipotential haemopoietic progenitor cells, suggesting that protein-protein interactions may play a significant role in the decision to commit to a particular lineage (Hu *et al.* 1997). Furthermore, alterations to the expression or activity of transcription factors modifies the balance of cells within the haemopoietic system through altered lineage commitment choices, blocked differentiation and inhibition of proliferation (Barreda and Belosevic 2001). It is therefore not surprising that several mechanisms act together to maintain the activity and expression of transcription factors at appropriate levels. These are redundancy, synergy and antagonism.

1.6.2.1.1.1 Redundancy

Similar to cytokines and cytokine receptors, transcription factors display functional redundancy to ensure differentiation along key haemopoietic pathways (Barreda and Belosevic 2001). One particular example of transcription factor redundancy can be found within the C/EBP family, where lipopolysaccharide-inducible expression of the genes encoding IL-6 and monocyte chemoattractant protein-1 can be mediated by C/EBPα, C/EBPβ, and C/EBPδ family members (Hu *et al.* 1998). C/EBP redundancy has also been observed in regard to the expression of the M-CSF receptor and has been attributed to similarity in bZIP domains of the C/EBP transcription factor molecules (Hu *et al.* 2000).

1.6.2.1.1.2 Cooperative or Synergistic Interactions

The upregulation of a common set of target genes through cooperative interactions between 2 or more transcription factors is another mechanism by which gene transcription can be controlled. For example, the activity of the neutrophil elastase gene promoter, which is expressed in immature myeloid cells, is positively and cooperatively regulated by PU.1, C/EBP and c-Myb (Oelgeschlager *et al.* 1996). Transfection of cells with each transcription factor alone increased activation of the neutrophil elastase promoter 5-fold, whereas transfection of the same cell line with PU.1, c-Myb and C/EBP together activated the neutrophil elastase promoter 17-fold (Oelgeschlager *et al.* 1996).

A much more dramatic example of synergistic activation comes from cotransfection studies where the transcription factors AML-1 (acute myelogenous leukaemia –1) and C/EBP significantly increased the activity of the M-CSF receptor promoter (Zhang *et al.* 1996). When C/EBP or AML-1 was transfected alone, there was no significant change to M-CSF promoter activity. However, when AML-1 and C-EBP were cotransfected, a 22-fold induction of M-CSF receptor promoter activity was detected, an effect that was found to be dependent on an intact DNA binding site (Zhang *et al.* 1996).

1.6.2.1.1.3 Antagonism

Negative interactions between transcription factors provides another means of haemopoietic regulation. For example, cross-antagonism between GATA-1 and PU.1 blocks differentiation in lineages where downregulation in the expression of these

transcription factors is normally associated with differentiation. The mechanism of corepression is unique to each transcription factor and plays a critical role in the decision of a stem cell to commit to either the erythroid or myeloid lineages (Zhang *et al.* 1999; Zhang *et al.* 2000).

The antagonistic model suggests that the relative abundance of each transcription factor may dictate the lineage decision of multipotent cells. In uncommitted cells, equivalent levels of each transcription factor would prevent either transcription factor from activating its target genes. Unilineage commitment occurs when the level of one transcription factor increases relative to another (Cantor and Orkin 2001). This cross antagonistic model of lineage commitment is consistent with the observation of multilineage gene expression by HSC prior to commitment to the erythroid or granulocytic lineages (Hu *et al.* 1997). When the transcriptional programs of multipotent single cells were examined using RT-PCR marked heterogeneity was observed in the gene expression profile of the cells examined. Most noteworthy was the coexpression of the β-globin (erythroid) and myeloperoxidase (myeloid) genes by 37% of the cells tested, providing direct evidence that in multipotential cells, several different lineage-associated programs of gene activity are primed prior to unilineage commitment (Hu *et al.* 1997).

Other cases of antagonistic interactions have been identified and provide strong support for functional interactions between transcription factors. For example, the c-Myb promoter is negatively regulated by PU.1 and, in cells constitutively expressing PU.1, G-CSF treatment is associated with a decrease in c-Myb mRNA levels (Bellon *et al.* 1997). These results are consistent with the temporal patterns of c-Myb and PU.1 expression, with c-Myb being expressed in haemopoietic progenitor cells when PU.1 expression is low. C-Myb levels decrease as cells differentiate which is in contrast to an upregulation in PU.1 expression (Bellon *et al.* 1997).

1.6.2.2 Notch Receptors & the Regulation of HSC Differentiation

Notch was initially identified in 1919 as a gene in which haplo-insufficiency causes "notches" at the wing margin in flies (reviewed in Kojika and Griffin 2001). Since then, it has become clear that Notch is essential for the normal development of many other tissues

in the fly including muscle, the peripheral nervous system and the eye (Kojika and Griffin 2001). Following the identification of Notch1 in normal bone marrow haemopoietic progenitor cells, considerable evidence has also emerged that supports a role for Notch in the mediation of cell fate decisions and self-renewal of progenitors during haemopoiesis (Milner *et al.* 1994).

The Notch genes encode a family of large transmembrane proteins that are thought to function as cell surface receptors mediating signals between cells located in close proximity of each other. Four Notch genes that show structural homology have been identified in vertebrates to date, and include Notch1/TAN-1, Notch2, Notch3 and Notch4/int-3 (Weinmaster *et al.* 1992; Kojika and Griffin 2001). The role of Notch receptors in myeloid differentiation is still controversial, although experiments tend to suggest Notch may inhibit the differentiation of immature myeloid precursor cells rather than promote differentiation (reviewed in Kojika and Griffin 2001).

Three Notch ligands have so far been identified in mammals, and are termed Delta (chromosome 6), Jagged1 (chromosome 20) and Jagged2 (chromosome 14) (Gray *et al.* 1999; Deng *et al.* 2000). Jagged1 expression appears to be confined to particular cell types such as stromal cells, mast cells, megakaryocytes and uncommitted stem cells, while Delta is expressed widely on haemopoietic cells and stromal cells, and Jagged2 on thymocytes and stromal cells (Li *et al.* 1998; Varnum-Finney *et al.* 1998; Kojika and Griffin 2001). While it appears that all 3 ligands bind to at least Notch1 and Notch2, it is unknown whether different receptor-ligand combinations result in different cell signals (Kojika and Griffin 2001). Ligand binding is essential for Notch activation and the fact that the ligands are also cell transmembrane receptor proteins suggests that signalling is dependent on cell-cell contact (Fehon *et al.* 1990; Kojika and Griffin 2001). These receptors provide a pathway for signal transduction from the cell surface to the nucleus and thereby allow the direct influence of gene expression on neighbouring cells (Milner and Bigas 1999).

It is probable that during haemopoietic development Notch signalling cooperates with, or modifies, the effects of various cytokine signals and/or transcription factors, to control cell fate and to maintain a pool of cells in an undifferentiated state (reviewed in Kojika and

Griffin 2001). It should also be noted that the effects of Notch1 and Notch2 may vary amongst individual cells considering that the effects of these receptors on cells is likely to be influenced by the levels of Notch expressed, the level of Notch-ligand expressed on neighbouring cells, and the maturational state of the cell (Kojika and Griffin 2001).

1.7 Dysregulated HSC Differentiation: Chronic Myeloid Leukaemia

In some instances cells escape the controls that normally limit their differentiation and proliferation. Myeloproliferative disorders (MPD) are chronic malignant conditions that originate in the haemopoietic stem cell pool. They involve overproduction of cells of the myeloerythroid lineage and include diseases such as polycythemia vera, idiopathic hypereosinophilic syndrome, myelofibrosis and chronic myeloid leukaemia (Cortes and Kantarjian 2004). Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of HSC that involves the expansion and premature release of progenitor cells at various stages of maturity and is distinguished from other MPD by the presence of a unique chromosomal abnormality known as the Philadelphia chromosome (Cortes and Kantarjian 2004). It can involve myeloid, erythroid, megakaryocytic, monocytic, and B cell lineages, and accounts for 15% of adult leukaemic cases (Faderl *et al.* 1999).

CML follows a triphasic course, and diagnosis is typically made during chronic-phase where there are elevated levels of mature granulocytes that retain the capacity to carry out biological function. Although chronic phase CML is readily controlled using cytotoxic agents in most patients, within 5 years of the initial diagnosis, the disease invariably progresses to accelerated phase and blast crisis where immature cells are released from the bone marrow and circulate throughout the body (Deininger *et al.* 2000). While the Philadelphia chromosome is generally the only recognizable cytogenetic abnormality in newly diagnosed CML patients, the evolution to blast crisis is often associated with the accumulation of secondary chromosomal abnormalities (Kabarowski and Witte 2000). It is important to note that while this leukaemic clone of cells dominates, a population of normal HSC coexists and is detectable in the bone marrow of CML patients (Coulombel *et al.* 1983).

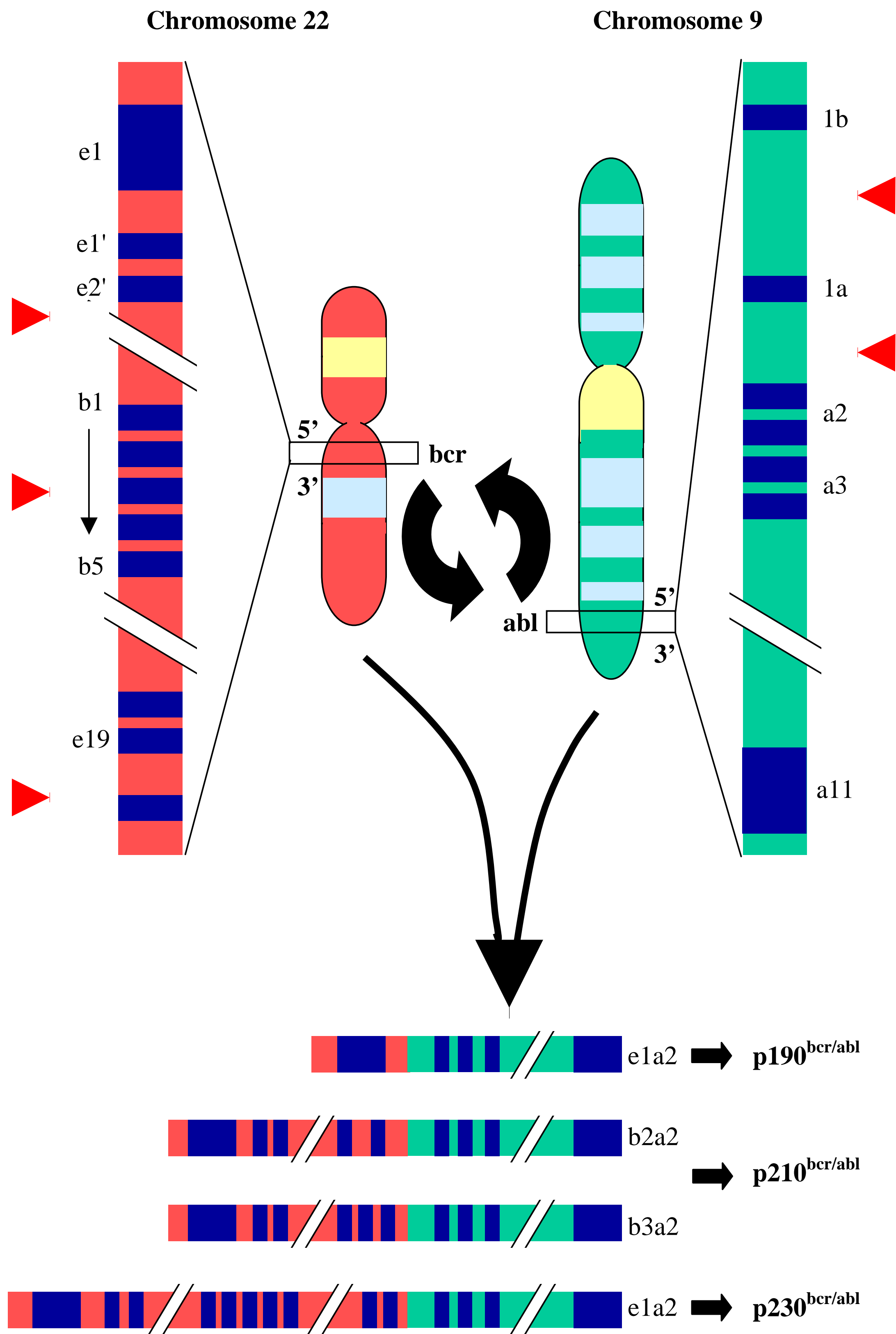
1.7.1 The Philadelphia Chromosome

The human c-Abl gene is located on chromosome 9, and is named after the Abelson virus which contains the viral oncogene v-Abl and causes leukaemia in mice (Abelson and Rabstein 1970; Jhanwar *et al.* 1984)]. It encodes a non-receptor tyrosine kinase that is ubiquitously expressed, and plays a major role in regulating cell cycle progression (Van Etten 1999). In quiescent cells the activity of *Abl* is controlled by binding of the protein to a complex that contains the retinoblastoma (Rb) protein. Once cells progress from G1 to S phase of the cell cycle, Rb becomes phosphorylated and dissociates from Abl, enabling Abl itself to become phosphorylated and activated and affect the transcription of target genes (reviewed in Holyoake 2001a). The bcr gene located on chromosome 22 is also ubiquitously expressed and although its function still remains to be elucidated, a role in cell cycle progression has been postulated (Groffen *et al.* 1984; Wetzler *et al.* 1995; Holyoake 2001a).

The hallmark of CML, seen in 95% of cases, is the Philadelphia (Ph) chromosome, and arises from a reciprocal translocation between the long arms of chromosomes 9 and 22 (Rowley 1973). This translocation adds the 3' segment of the Abl gene on chromosome 9 to the 5' segment of bcr on chromosome 22, and results in the generation of the hybrid gene bcr-abl (Rowley 1973; Groffen *et al.* 1984; Shtivelman *et al.* 1986). This bcr-abl gene is transcribed into a chimeric bcr-abl mRNA, and encodes for a nonreceptor tyrosine kinase that displays constitutive and deregulated activity in comparison to the parent c-Abl protein (Groffen *et al.* 1984; Konopka *et al.* 1985; Ben-Neriah *et al.* 1986). The subcellular location of the protein also differs to that of the parent c-Abl protein, in that bcr-abl is found exclusively in the cytoplasm (Dhut *et al.* 1991). As a consequence of these 2 alterations, bcr-abl phosphorylates several cellular substrates, activates multiple signal transduction pathways, and pushes the balance between self-renewal and differentiation divisions sharply towards differentiation (Faderl *et al.* 1999; Holyoake 2001a).

Several breakpoint locations have been identified along the bcr gene, and result in the generation of different lengths of bcr-abl mRNA depending on the break points involved (Faderl *et al.* 1999) (Figure 1.2). This mRNA is transcribed into chimeric protein products

Figure 1.2. Translocation of chromosomes 9 and 22 in CML. The Philadelphia chromosome results from a translocation of 3' abl segments on chromosome 9 to 5' bcr segments on chromosome 22. Breakpoints (indicated by red arrowheads) typically occur 5' of exon a2 on the abl gene, while various breakpoint locations have been identified along the bcr gene. This results in the generation of messenger RNA molecules of different lengths that are transcribed into chimeric protein products (p190, p210 and p230) of variable molecular weights. Adapted from Faderl *et al.* 1999.



Chromosome 22
Chromosome 9
e1
e1'
e2'
b1
b5
e19
5'
bcr
3'
abl
5'
3'
1b
1a
a2
a3
a11
e1a2
p190bcr/abl
b2a2
p210bcr/abl
b3a2
e1a2
p230bcr/abl

that consequently display variable molecular weights (p190, p210 and p230), and presumably different functions (Faderl *et al.* 1999). In 95% of CML cases, p210 mRNA is detected and implicated in the pathogenesis of the disease (Konopka *et al.* 1985; Ben-Neriah *et al.* 1986). The first evidence that implicated bcr-abl in the pathogenesis of leukaemia came from *in vivo* studies using bcr-abl transgenic mice, where progeny from p190 transgenic mice developed acute leukaemia (Heisterkamp *et al.* 1990).

1.7.2 Selective Advantage of CML Progenitors

The mechanisms behind the resultant accumulation and expansion of leukaemic progenitors are not fully understood, but include increased survival due to an anti-apoptotic effect (Bedi *et al.* 1994), a reduced effectiveness of proliferation inhibitors such as the macrophage inflammatory protein 1-α (Chasty *et al.* 1995), enhanced cell cycle activation (Kramer *et al.* 2001), and altered adhesion to stroma cells and extracellular matrix (Gordon *et al.* 1987). Various signalling pathways are linked to these effects, and include the Ras and, in turn, Erk-kinase and Jun-kinase pathways, CRKL-linked pathways, Jak-STAT (particularly STAT5) and the Src pathway (Gesbert and Griffin 2000; Holyoake 2001a).

The precise effect of bcr-abl expression on the proliferation of CML progenitor cells remains controversial. While the growth of CML progenitors has been demonstrated to be similar to that of normal progenitor cells (Bedi *et al.* 1994; Thiele *et al.* 1997), contradictory results have been observed and suggest that bcr-abl stimulates proliferation (Lepine and Messner 1983; Cortez *et al.* 1997; Jonuleit *et al.* 1998). In a further study using late progenitor cells ($CD34^{+}Lin^{+}$) from CML patients, these cells cycled at a slower rate than cells from normal donors. It was suggested from this study that CML is not a disease of uncontrolled proliferation, and rather involves a defect in the balance between self-renewal and maturation (Buckle *et al.* 2000).

The features of chronic phase CML do not correlate with an increase in cellular proliferation, and the discordant maturation model of Clarkson and Strife (1988) postulates that the increased myeloid mass in CML patients is due to increased numbers of cell divisions prior to terminal differentiation and increased cell survival. The authors also predicted that abnormal maturation may provide a background by which a further

mutational event in the progenitor cell(s) may occur, resulting in transition to accelerated phase or blast crisis (Strife and Clarkson 1988).

Recent interest has focussed on the observation that bcr-abl expressing cells display reduced or absent growth factor dependence (Bedi *et al.* 1994) and, once p210 cDNA was cloned from cells that had undergone transformation by bcr-abl, attempts were made to replicate CML *in vivo* and *in vitro*. *In vitro* experiments demonstrated that bone marrow cells infected with a retrovirus carrying bcr-abl displayed growth factor independence but were not tumorigenic (Gishizky and Witte 1992), while enforced expression of bcr-abl in a factor-dependent cell line rendered cells growth factor independent and tumorigenic (Daley and Baltimore 1988; Kabarowski *et al.* 1994). Transformation to complete growth factor independence was not immediate, and the level of gene expression was found to correlate with the transforming ability of bcr-abl (Gishizky and Witte 1992; Kabarowski *et al.* 1994). In addition, purified populations of very primitive bcr-abl$^+$ cells isolated from CML patients proliferated *in vitro* for several weeks in the absence of growth factors (Maguer-Satta *et al.* 1998). This effect was shown to involve an autocrine IL-3 and G-CSF mechanism (Jiang *et al.* 1999).

Altered adhesion of HSC to bone marrow stroma and extracellular matrix has also been implicated as a mechanism in malignant transformation by bcr-abl in CML (Deininger *et al.* 2000). CML progenitor cells express elevated levels of integrin receptors, and abnormal function of these receptors is associated with decreased adhesion to stroma and fibronectin (Gordon *et al.* 1987; Verfaillie *et al.* 1992). Abnormalities in integrin function in CML progenitor cells are believed to be a direct consequence of bcr-abl expression and may contribute to abnormal circulation and unregulated growth of leukaemic cells (Bhatia *et al.* 1999; Bhatia *et al.* 2001).

1.7.3 Identification of Quiescent CML Progenitor Cells

It has been suggested that a highly quiescent population of leukaemic stem cells exist in CML patients, and that these cells play a role in sustaining the disease. Two approaches have been used to demonstrate the isolation of this quiescent population that displays stem cell properties, and the fact that the quiescence was reversible suggests that this population

is indeed involved in maintenance of CML (Holyoake *et al.* 1999). The detection of these bcr-abl$^+$ quiescent HSC suggests that the expression of bcr-abl alone does not necessarily promote the entry of the cells into the cell cycle and that, to some degree, these cells retain normal responses to differentiative and proliferative signals (Kabarowski and Witte 2000). The entry of bcr-abl$^+$ progenitor cells into the quiescent state was found to be highest in the more primitive progenitor cells (CD38$^-$, CD45RA$^-$, CD71$^-$, HLA-Dr^{lo}), and was associated with a down regulation in IL-3 and G-CSF gene expression. Upon entry into the cell cycle, IL-3 gene expression was upregulated (Holyoake *et al.* 2001b). This suggests that bcr-abl confers a proliferative advantage over normal cells when cytokine concentrations are low (Holyoake *et al.* 2001b).

1.7.4 Treatment of CML

CML is almost uniformly fatal except in instances where bone marrow transplants are possible. Approximately one third of CML patients are eligible for allogeneic bone marrow transplantation, and the cure rate for those who survive the transplant is 70-90% (Carroll *et al.* 1997). For those remaining 65% of patients who are not suitable for bone marrow transplants, cytotoxic treatment including hydroxyurea, interferon or imatinib is the remaining option.

1.7.4.1 Interferon

Interferons (IFN) are glycoproteins produced by eukaryotic cells in response to antigens, such as viral infection or malignant disease, and bind to the IFN receptor (Faderl *et al.* 1999; Jonasch and Haluska 2001). They display anti-viral, anti-proliferative and anti-angiogenic effects and, while they also appear to specifically target CML cells, their precise mechanism of action remains unknown (Faderl *et al.* 1999). Treatment with interferon-α is more successful in prolonging survival than chemotherapy and can induce complete cytogenetic responses in 5-20% of CML patients. Unfortunately, this drug is also associated with serious side effects such as fatigue, anorexia and neuropsychiatric symptoms that have a major impact on the patient's quality of life (Jonasch and Haluska 2001).

1.7.4.2 Imatinib

The tyrosine kinase activity of the bcr-abl protein is essential for its transformation of cells, so it seems logical that treatment of CML should involve inhibition of this activity. Over one thousand cellular tyrosine kinases that may potentially play a role in cell survival, proliferation and/or oncogenesis have been identified. Two classes of tyrosine kinase inhibitors are currently being developed to target these tyrosine kinases, with one class targeting the tyrosine kinase ATP binding site, and the other class inhibiting the substrate binding site (Faderl *et al.* 1999).

The tyrosine kinase inhibitor, imatinib (Glivec, previously STI-571), is a 2-phenylaminopyrimidine derivative that has been shown to specifically inhibit the growth of bcr-abl expressing CML progenitors with minimal effects on the survival and growth of normal cells (Druker *et al.* 1996; Carroll *et al.* 1997). It functions as a potent ATP-competitive inhibitor of the Abl protein kinase, thereby inhibiting the kinase activity of bcr-abl and specifically inducing apoptosis in leukaemic cells (Gambacorti-Passerini *et al.* 1997) (Figure 1.3).

Imatinib is essentially inactive against serine/threonine-kinases and most tyrosine kinases, with exceptions including normal Abelson, platelet derived growth factor receptor (PDGFR), ARG and c-kit tyrosine kinases (Carroll *et al.* 1997; Buchdunger *et al.* 2000; Schindler *et al.* 2000; Okuda *et al.* 2001). The activation loop of most kinases controls the catalytic activity by switching between different phosphorylation states and, in fully active kinases, the activation loop is stabilised in an open conformation by phosphorylation on serine, threonine or tyrosine residues within the loop (Schindler *et al.* 2000). Although the conformation of the activation loop is similar in all known structures of active kinases, and imatinib targets a well conserved nucleotide binding pocket of Abl, this drug achieves high specificity by recognising a distinctive inactive conformation of the activation loop of Abl (Schindler *et al.* 2000).

Through targeting of c-kit, imatinib has also been used in the treatment of gastrointestinal stromal tumours (GIST) (Verweij *et al.* 2003). GISTs are characterised by expression of the transmembrane receptor tyrosine kinase, c-kit, and activating or gain-of-function

mutations in the c-kit gene have been identified in most GIST cases (Hirota *et al.* 1998; Nakahara *et al.* 1998; Sarlomo-Rikala *et al.* 1998). Since GISTs have a high rate of resistance to standard conventional chemotherapeutic agents, imatinib provides a novel approach to the treatment of these mesenchymal tumours by inhibiting ligand-independent c-kit phosphorylation, decreasing cellular proliferation and inducing apoptosis (Demetri 2001; Verweij *et al.* 2003).

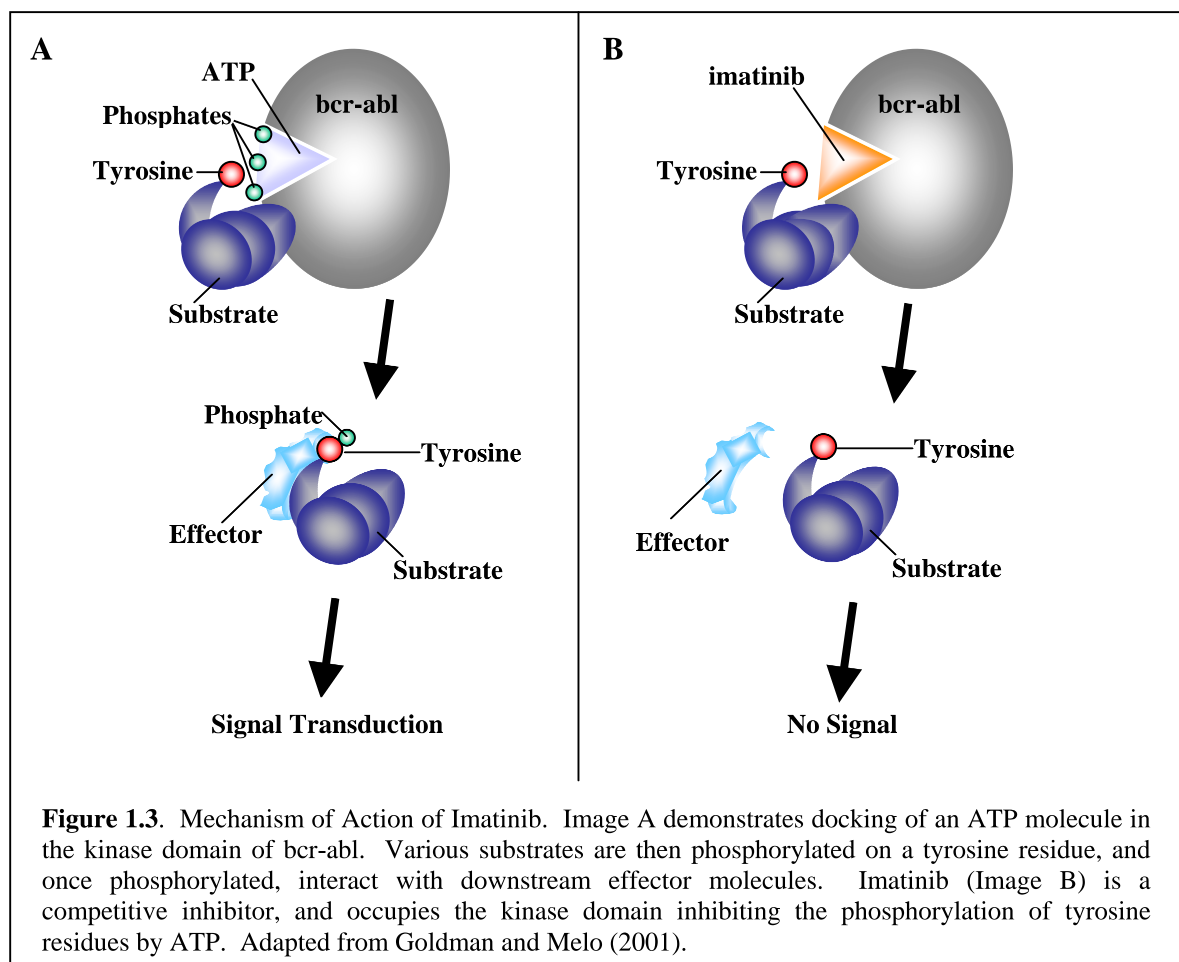


Figure 1.3. Mechanism of Action of Imatinib. Image A demonstrates docking of an ATP molecule in the kinase domain of bcr-abl. Various substrates are then phosphorylated on a tyrosine residue, and once phosphorylated, interact with downstream effector molecules. Imatinib (Image B) is a competitive inhibitor, and occupies the kinase domain inhibiting the phosphorylation of tyrosine residues by ATP. Adapted from Goldman and Melo (2001).

1.7.4.2.1 Resistance to Imatinib

While treatment with imatinib appears promising, bcr-abl$^+$ cells that are resistant to treatment by imatinib have emerged in patients that were initially responsive to the drug, and a proportion of patients are primarily refractory to imatinib (Barthe *et al.* 2001; Gorre *et al.* 2001; Hochhaus *et al.* 2001). *In vitro* findings in support of these observations have been made in 3 independent laboratories, where bcr-abl$^+$ cell lines cultured for long periods

of time in increasing concentrations of imatinib developed resistance to the drug (le Coutre *et al.* 2000; Mahon *et al.* 2000; Weisberg and Griffin 2000). The mechanisms of resistance were found to be diverse and varied across the different cell lines, but included an up-regulation of bcr-abl gene expression, reduced P-glycoprotein-mediated intake of imatinib, (le Coutre *et al.* 2000; Mahon *et al.* 2000; Weisberg and Griffin 2000), and MDR1 gene over-expression causing drug efflux (Mahon *et al.* 2003).

In patient trials, the most common mechanism underlying this clinical resistance appears to be mutations in bcr-abl that may impair imatinib binding**,** and increased levels of bcr-abl through gene amplification (Gorre *et al.* 2001; Branford *et al.* 2002; Hochhaus *et al.* 2002; Shah *et al.* 2002; Branford *et al.* 2003). Other mechanisms of resistance that may be a result of genetic mutations include decreased intracellular levels of imatinib and intrinsic changes in the kinase that affect imatinib binding or kinase activity (Ross and Hughes 2004).

It appears that one specific mutation to the bcr-abl kinase domain is not responsible for conferring imatinib resistance in all patients, as a number different mutations have so far been identified that are associated with a poor prognosis (Barthe *et al.* 2001; Gorre *et al.* 2001; Branford *et al.* 2003). Current studies are addressing whether different mutations segregate with different clinical phenotypes or with different patterns of clinical resistance, as the emergence of mutations at amino acid positions 250-255 which form the adenosine triphosphate-binding loop (P-loop) appear to carry a particularly poor prognosis (Branford *et al.* 2003). It seems that in some cases, mutations conferring imatinib resistance are present in a small proportion of leukaemic cells prior to therapy and are positively selected during imatinib treatment (Ross and Hughes 2004).

Several strategies to overcome resistance to imatinib are currently being investigated. One particular approach is imatinib dose escalation to overcome amplification and/or over-expression of bcr-abl (Kantarjian *et al.* 2003). In this study, CML patients who relapsed on a daily dose of 400mg imatinib were treated with 800mg daily, and patients who relapsed on a daily dose of 300mg were treated with 600mg daily. Fifty-six percent of patients who had cytogenetic resistance or relapse achieved a complete or partial cytogenetic response,

suggesting that a higher dose of imatinib may overcome poor responses to conventional doses (Kantarjian *et al.* 2003).

In addition to imatinib resistance in CML patients, additional novel and specific c-kit mutations are emerging in GIST patients that confer drug resistance (Chen *et al.* 2004). In a study of 12 GIST patients with initial responses to imatinib, rapidly progressive metastatic foci that were resistant to imatinib treatment were observed in 5 patients within 31 months of treatment, and quiescent residual GISTs persisted in seven patients (Chen *et al.* 2004). In the patients with resistant metastatic foci, identical novel c-kit missense mutations were observed that were strongly associated with imatinib resistance (Chen *et al.* 2004).

1.7.4.2.2 Effect of Imatinib on Normal Haemopoiesis

Clinical trials of imatinib for the treatment of CML and GIST have demonstrated that imatinib can induce complete haematological and cytogenetic responses in a large proportion of CML patients, and prevent disease progression in GIST patients, with minimal side effects (Druker *et al.* 2001; Sawyers *et al.* 2002; Verweij *et al.* 2003). The most commonly reported side effects are haematological and include neutropenia, thrombocytopenia, anaemia and granulocytopenia. It is not clear, however, if these cytopenias also result from suppression of normal haemopoiesis.

In CML patients the myelosuppression that arises as a result of imatinib treatment has been attributed to the pharmacological effect of imatinib on leukaemic cells and a lag time before normal haemopoietic stem cells recover from suppression by the malignant clone (Sawyers *et al.* 2002). Data from GIST patients treated with imatinib suggest that imatinib directly suppresses normal haemopoiesis, if it is assumed that GIST has no effect on haemopoiesis in these patients. In a phase II study performed on 51 patients who had GIST or other soft tissue sarcomas (STS) and received 400mg imatinib daily, 92% of those patients displayed anaemia and 47% displayed granulocytopenia (Verweij *et al.* 2003). Most of these cytopenias were only mild-moderate in severity however, and no GIST or STS patients were withdrawn from treatment.

Since imatinib is still in its early stages of use as a drug to treat CML and GIST, the implications of long-term treatment are unknown. Reports are beginning to emerge that show that normal haemopoietic cells are affected by imatinib (Appel *et al.* 2003; Bartolovic *et al.* 2003; Dewar *et al.* 2003). This inhibitory effect has been observed in normal CD34$^+$ progenitor cells and dendritic cells, and has been found to be independent of c-kit signalling (Appel *et al.* 2003; Bartolovic *et al.* 2003; Dewar *et al.* 2003).

1.8 Summary & Project Aims

Haemopoietic stem cells are crucial for life as many essential cells, such as myeloid and erythroid cells, are short-lived and require continual replenishment. This review illustrates the complexity of haemopoiesis and the wide range of factors involved in its regulation. Current evidence suggests that the decision of a stem cell to self-renew or differentiate is an intrinsic property of the cell and therefore under stochastic control. In this model, the balance of transcription factors plays an important role in the decision of haemopoietic progenitor cells to commit to a particular lineage, and cytokines merely support the survival of these cells.

While some insight has been gained into regulation of haemopoiesis, it is still unclear how differentiation decisions are made, and how leukaemogenesis modifies this process. CML is a clonal myeloproliferative disorder of HSC which occurs as a result of the formation of a fusion protein known as bcr-abl, and involves the expansion and premature release of progenitor cells at various stages of maturity. The mechanisms behind the accumulation and expansion of leukaemic progenitors are not fully understood, although they are thought to include decreased apoptosis (Bedi *et al.* 1994), enhanced cell cycle initiation (Kramer *et al.* 2001) and altered adhesion to stroma cells and the extracellular matrix (Gordon *et al.* 1987). The resultant effect of bcr-abl on the proliferation of CML cells remains controversial, however, and a limited understanding of the factors that control HSC behaviour under normal conditions impedes identification of abnormalities in the differentiation and proliferation of CML cells.

Recent development of the tyrosine kinase inhibitor imatinib, which is targeted to the ATP binding domain bcr-abl, has vastly improved cytotoxic treatment of CML and is associated

with few side effects. Imatinib has shown relative specificity for CML cells, however it is becoming increasingly apparent that this drug also has an inhibitory effect on normal haemopoiesis. This observation may account for the myelosuppression observed in some patients treated with imatinib, and identification of further drug targets offers the possibility of extending the use of this drug for other diseases involving dysregulated cell growth.

In order to investigate these issues, the following aims were addressed:

- To determine whether the differentiation of normal $CD34^+$ cells, with respect to acquisition of phenotype, correlated to cell division events.

- To identify perturbations to the normal program of $CD34^+$ cell differentiation in $CD34^+$ cells from CML patients.

- To determine whether therapeutic concentrations of imatinib inhibit normal haemopoiesis, and if so,
    - to identify specific cell types inhibited by imatinib;
    - to identify further tyrosine kinases inhibited by imatinib.

Chapter 2:

MATERIALS & METHODS

2.1 Suppliers of Commonly Used Reagents

Table 2.1: Commonly Used Reagents & their Suppliers

Reagent	Supplier	Catalogue Number
2-ethoxyethanol	APS Fine Chemicals	211-500M
α-Naphthyl butyrate	Sigma®	N-8000
β-Mercaptoethanol	Sigma®	M-6250
Acrylamide (40% bis solution)	Bio-Rad	161-0148
Bacto™ Agar	Difco	214010
Bovine serum albumin (BSA) powder (Fraction V)	Sigma®	A-9418
Disodium hydrogen phosphate (Na_2HPO_4)	Aldrich	219886-500g
Dimethyl sulphoxide (DMSO)	APS Fine Chemicals	747-500mL
DNase	Sigma®	D-5025
Dulbecco's modified Eagle's medium (DMEM)	JRH Biosciences	51444-500MA
Dynabead CD34 Progenitor cell selection system	Dynal	113.01 / M-450
EDTA	APS Fine Chemicals	180/500g
Ethanol (analytical grade)	AnalaR®, BDH	10107.2500P
Fast Blue BB Salt	Sigma®	F-3378
Fast Garnet GBC	Sigma®	F-8761
Ficoll-Hypaque (Lymphoprep 1.077 g/dL)	Axis-shield, Oslo, Norway	1113069
Foetal calf serum (FCS)	Hyclone	SH30072-03
Formaldehyde	AnalaR®, BDH	10113
Glucose	Sigma®	G-7528
Glutaraldehyde	Sigma®	G-6403
Hanks balanced salt solution (HBSS)	JRH Bioscience	55021-500M
HEPES (1M)	JRH Biosciences	59205-100M
Holo-transferrin	Sigma®	T-0665

Table 2.1(Continued): Commonly Used Reagents & their Suppliers

Reagent	Supplier	Catalogue Number
Insulin	Novo Nordisk Pharmaceuticals	Actrapid® 10mL
Iscove's modified Dulbecco's medium (IMDM)	JRH Biosciences	51471-500M
L-asparagine	Sigma®	A-0884
L-glutamine	JRH Biosciences	59202-100M
Lauryl sulphate (SDS)	Sigma®	L-4509
Lipoprotein (low density)	Sigma®	L-7914
Luxol Fast Blue MBS	Gurr®, BDH	34044 3P
Methyl violet	AnalaR® BDH	34033
N,N-Dimethylformamide	APS Fine Chemicals	2217-500M
Naphthol AS-D Chloroacetate	Sigma®	N-0758
Percoll (ρ = 1.130g/mL)	Amersham	17-0891-02
Penicillin G (5000 U/mL) - Streptomycin sulphate (5000 μg/mL)	CSL Biosciences	05081901
Potassium dihydrogen orthophosphate (KH_2PO_4)	AnalaR® BDH	10203.4B
Sodium chloride	APS Fine Chemicals	465-2.5kg
TMB Liquid Substrate System for ELISA	Sigma®	T-0440
Tris (Sigma-7-9®)	Sigma®	T-1378
Trypan blue (0.4%)	Sigma®	T-8154
Tween-20	Sigma®	P-9416
Urea	APS Fine chemicals	817-5kg

2.2 Solutions, Buffers & Media for Cell Culture

2.2.1 0.66% Agar

Bacto™ Agar	0.33g
Milli-Q® water	50.0mL

The agar and Milli-Q® water (Millipore Corporation, USA) were combined in a 100mL bottle and autoclaved at 121°C for 30 minutes on a fluid cycle. Approximately 1 hour after the autoclave cycle was complete, the solution was swirled to evenly distribute the agar. The 0.66% agar solution was stored at room temperature until required. For use in colony assays, the agar was melted by microwaving on high for 30 seconds, swirling thoroughly, and microwaving for a further 15 seconds. The agar was then retained at 41°C on a heating block.

2.2.2 Agar- Iscove's Modified Dulbecco's Medium (A-IMDM)

FCS	25.0mL
2x IMDM	25.0mL
L-glutamine	500µL

Constituents were combined and sterilised using a 0.2µm single use syringe filter (Sartorius Minisart) on the day of use. For use in colony assays, the solution was held at 37°C in a water bath.

2.2.3 Assay Buffer for IL-3/IL-6 ELISA (PBS/0.5% BSA/0.1% Tween-20)

PBS	500mL
BSA	2.5g
Tween-20	500µL

The BSA was dissolved slowly in PBS with the aid of a magnetic stirrer. Tween-20 was then added, and the solution sterilised using a 0.2µm bottle top filter (Vacu Cap®, PALL Life Sciences). The solution was stored at 4°C.

2.2.4 Assay Buffer for M-CSF ELISA (PBS/1.0% BSA)

PBS	500mL
BSA	5.0g

The BSA was dissolved slowly in PBS with the aid of a magnetic stirrer. The solution sterilised using a 0.2µm bottle top filter and stored at 4°C.

2.2.5 Blocking Buffer (HBSS/0.8% BSA/ 4% NHS/ 5% FCS)

HBSS	500mL
BSA powder	4.0g
Normal human serum	20.0mL
FCS	25.0mL

The BSA was dissolved slowly in HBSS with the aid of a magnetic stirrer. The HBSS/BSA solution was further supplemented with normal human serum and FCS. The solution was sterilised with a 0.2µm bottle top filter and stored at 4°C.

2.2.6 Double-Strength Iscove's Modified Dulbecco's Medium (2x-IMDM)

IMDM Powder	1 portion (17.67g)
L-glutamine	0.4g
L-asparagine	0.2g
$NaHCO_3$	6.048g
Mill-Q® water	

IMDM was reconstituted from powder using 400mL Milli-Q® water, and supplemented with L-glutamine, L-asparagine and $NaHCO_3$. The ingredients were dissolved at room temperature with the aid of a magnetic stirrer, and the volume adjusted to 500mL using Milli-Q® water. The solution was sterilised using a 0.2µm bottle top filter and 25mL aliquots stored at –20°C.

2.2.7 Dulbecco's Modification of Eagle's Medium$^+$ (DMEM$^+$)

DMEM	500mL
FCS	50.0mL
L-glutamine (200mM)	5.0mL
Penicillin 5000U/mL /	
Streptomycin 5000µg/mL	5.0mL

DMEM was supplemented with FCS, L-glutamine and penicillin/streptomycin and stored at 4°C.

2.2.8 Flow Cytometry Fixative (FACS Fixative)

PBS	500mL
40% w/v Formaldehyde	5.0mL
D-glucose	10.0g
NaN_3	0.1g

The formaldehyde and glucose were added to the PBS, and the solution stored indefinitely at 4°C.

2.2.9 3% Glutaraldehyde Fixative

Glutaraldehyde	15.0mL
Milli-Q® water	485mL

Glutaraldehyde and Milli-Q® water were combined and stored indefinitely at room temperature.

2.2.10 HHF (HBSS/10mM HEPES/5% FCS)

Hanks Balanced Salt Solution	500mL
HEPES (1M)	5.0mL
FCS	25mL

Sterile HEPES and FCS were added to Hanks Balanced Salt Solution (HBSS). The solution was stored at 4°C.

2.2.11 Imatinib (10mM stock)

Imatinib powder	0.051g
Milli-Q® water	8.648mL

Imatinib (Novartis, Basel, Switzerland) was dissolved in Milli-Q® water and the solution sterilised using a 0.2µm single use syringe filter. The solution was stored indefinitely at 4°C. A titration range of 0.3µM-50.0µM imatinib was selected for use in this study, with administered therapeutic doses of 400mg and 600mg imatinib daily equating to an *in vitro* concentration of approximately 1.0-1.9µM and 3.7µM imatinib respectively (Bakhtiar *et al.* 2002; le Coutre *et al.* 2004).

2.2.12 IMDM/1% BSA

IMDM	500mL
BSA	5.0g

The BSA was dissolved slowly in IMDM using a magnetic stirrer. The solution was sterilised using a 0.2µm bottle top filter and stored at 4°C.

2.2.13 Isolation Buffer (IB) (HBSS/2% BSA/DNase)*

HBSS	500mL
BSA	10.0g
DNase*	25000 units

The BSA was dissolved slowly in HBSS with the aid of a magnetic stirrer. The solution was sterilised using a 0.2μm bottle top filter and stored at 4°C. *For cryopreserved and CML cell suspensions, DNase was added to the solution prior to filtration.

2.2.14 MACS CD34$^+$ Buffer (HBSS/0.5% BSA/2mM EDTA)

HBSS	449mL
BSA	2.5g
EDTA (1M stock)	1.0mL

BSA was dissolved slowly in HBSS using a magnetic stirrer. The EDTA was added to the buffer, and the solution sterilised using a 0.2μm bottle top filter. The buffer was stored at 4°C.

2.2.15 MACS Monocyte Buffer (HBSS/0.5% BSA/10% FCS/2mM EDTA)

HBSS	449mL
BSA	2.5g
FCS	50mL
EDTA (1M stock)	1.0mL

BSA was dissolved slowly in HBSS using a magnetic stirrer. The FCS and EDTA were added to the buffer, and the solution sterilised using a 0.2μm bottle top filter. The buffer was stored at 4°C.

2.2.16 2-Mercaptoethanol (1M)

β-mercaptoethanol (14.27M)	700μL
HBSS	9.3mL

β-mercaptoethanol was diluted in HBSS and stored at –20°C. This 1M stock solution was further diluted 1:10 in HBSS to give a working concentration of 0.1M.

2.2.17 Normal Human Serum

Normal human serum (NHS) was obtained from the Red Cross Blood Service and was heat inactivated at 56°C for 45 minutes. Ten millilitre (10mL) aliquots were stored at –20°C.

2.2.18 Percoll (ρ = 0.99g/mL)

Percoll (ρ = 1.130g/mL)	40mL
10x PBS	6.6mL

The Percoll and PBS were combined and the pH adjusted to 7.0 with 0.1M HCl. The solution was sterilised using a 0.2μm single use syringe filter, and stored at 4°C for a maximum of 2 months.

2.2.19 0.067M Phosphate Buffer, pH 6.3

KH_2PO_4	7.0g
Na_2HPO_4	2.2g
Milli-Q® water	

KH_2PO_4 and Na_2HPO_4 were dissolved in 950mL of Milli-Q® water, with the aid of a magnetic stirrer. The pH of the solution was adjusted to 6.3 using 1M HCl or 1M NaOH, and the volume adjusted to 1.0L using Milli-Q® water.

2.2.20 0.067M Phosphate Buffer, pH 7.4

KH_2PO_4	1.74g
Na_2HPO_4	7.68g
Milli-Q® water	

KH_2PO_4 and Na_2HPO_4 were dissolved in 950mL of Milli-Q® water, with the aid of a magnetic stirrer. The pH of the solution was adjusted to 7.4 using 1M HCl or 1M NaOH, and the volume adjusted to 1.0L using Milli-Q® water.

2.2.21 Serum Deprived Medium (SDM)

IMDM/1% BSA	10.0mL
L-glutamine (200mM)	100µL
Insulin (1mg/mL)	100µL
Transferrin (20mg/mL)	100µL
Lipoprotein (low density) (12.5mg/mL)	40µL
2-mercaptoethanol (0.1M)	5µL

The constituents were combined, and sterilised using a 0.2µm single use syringe filter. This resulted in a solution of IMDM/1% BSA supplemented with 2mM L-glutamine, 200µg/mL transferrin, 10µg/mL insulin, 10^{-4}M β-mercaptoethanol, and 50µg/mL low density lipoproteins. The solution was used fresh or stored at –20°C.

2.2.22 Thaw Solution

IMDM	400mL
FCS	100mL
L-asparagine	0.1g
L-glutamine (200mM)	5.0mL
DNase	25000 units

L-asparagine was dissolved in IMDM with the aid of a magnetic stirrer. The remaining constituents were then added, and the solution sterilised using a 0.2µm bottle top filter. The solution was stored at 4°C, and heated to 37°C in a water bath prior to use.

2.2.23 Urea Saturated Ethanol

Urea was added to ethanol at room temperature until a saturated solution was attained. The solution was stored indefinitely at room temperature.

2.2.24 White Cell Fluid

Acetic acid	2.0mL
Milli-Q® Water	98mL
Methyl Violet	Few crystals

Acetic acid was added slowly to Milli-Q® water. The methyl violet (Gurr®, BDH) was then added, and the solution mixed well to ensure that the crystals completely dissolved. The solution was filtered using a 0.2μm bottle top filter, and stored indefinitely at room temperature.

2.3 Cell Culture Techniques

All appropriate tissue culture techniques were performed in a Class two "biohazard" laminar flow hood (Gelman Sciences).

2.3.1 Maintenance of Cell Lines

Non-adherent cell lines were maintained at a cell density between $5.0x10^4$ and $1.0x10^6$/mL in $25cm^2$, $75cm^2$ or $150cm^2$ tissue culture flasks (Greiner). Media was prewarmed to 37°C prior to use.

Adherent cell lines were maintained under sub-confluent conditions. Near-confluent cells were harvested by aspirating the supernatant, washing the cells once with PBS, and incubating the cells with trypsin for 2-5 minutes at 37°C. Cells were dislodged by tapping the flask firmly, and the trypsin activity inhibited by the addition of a 5-10% protein solution (eg $DMEM^+$). Cell cultures were re-established by seeding fresh culture flasks with a cell suspension that had been diluted between 1/10 and 1/30.

2.3.1.1 FDC-P1

The murine factor-dependent cell line, FDC-P1, grew as a suspension culture in $DMEM^+$ supplemented with 1/2000 dilution of murine IL-3.

2.3.1.2 Psi-2 (ψ2)

The adherent ecotropic packaging cell line ψ2 was maintained in $DMEM^+$ under sub-confluent conditions.

2.3.2 Cryopreservation of Cells

Cells were cryopreserved in a FCS solution containing the cryoprotectant, dimethyl sulphoxide (10% (v/v) final concentration) (DMSO). Cells were suspended in FCS (neat) and, immediately prior to freezing, an equal volume of ice-cold 20% (v/v) DMSO in FCS was added to the cells drop-wise with constant mixing. The cell suspension was transferred quickly to cryoampoules (Nalgene®) and the samples stored in liquid nitrogen (-196°C). The final concentration of cells was a maximum of 1.0×10^7/mL.

2.3.3 Thawing of Cryopreserved Cells

Cells were removed from liquid nitrogen and thawed rapidly in a 37°C water bath. The thawed cell suspension was transferred quickly to a 50mL polypropylene conical tube (Falcon), and approximately 15mL of thaw solution (warmed to 37°C) was added drop wise with constant mixing. The sample volume was then increased to 30mL with thaw solution and the cells pelleted by centrifugation at 420g for 5 minutes in a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge. The supernatant was aspirated and the cells washed a further 2 times with HHF to remove any residual DMSO.

2.3.4 Cell Washing

For large volumes of cell suspensions, cells were pelleted by centrifugation at 420g for 5 minutes at 4°C using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge. Alternatively, cell washing following antibody staining was routinely performed at room temperature by centrifugation for 2 minutes at 815g in a DiaCent-12 mini centrifuge (DiaMed). Supernatants were removed by aspiration, and cells resuspended in appropriate medium.

2.3.5 Cell Counts and Viability

Cell concentration was determined by diluting the cell suspension in white cell fluid (WCF), while cell viability was assessed by diluting samples with 0.4% trypan blue solution. Ten microlitres (10µL) of these suspensions were transferred to a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell concentration and viability calculated accordingly.

2.3.6 Immunofluorescent Staining & Flow Cytometric Analysis

Flow cytometric analysis was performed using an Epics®-XL-MCL flow cytometer (Beckman Coulter). Cell populations were analysed on the basis of their forward and side light scattering properties (indicative of cell size and granularity respectively) and/or the fluorescence intensity of various fluorochromes.

2.3.6.1 Antibody Staining for Flow Cytometric Analysis

A known number of cells were transferred to 5mL round bottom polystyrene tubes (Falcon) and suspended in a minimum volume (~200µL) of HHF. Appropriate antibodies (see table 2.2) were added to tubes at a concentration of 10µL/10^6 cells, unless otherwise stated, and incubated for a period of 45 minutes on ice in the dark. Cells were then washed twice prior to the addition of a secondary antibody conjugate, or prior to fixation in FACS fix. Fixed samples were stored at 4°C in the dark until analysis was performed. The fluorescence intensity of cell suspensions was examined using an Epics®-XL-MCL flow cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter). Control tubes stained with an isotype-matched control were included in all experiments, and were used to define the cut-off point for positive/negative staining.

2.3.6.2 Carboxyfluorescein Diacetate Succinimidyl Ester Labelling of Cells

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a fluorescein derivative that is cell permeant and non-fluorescent. The cleavage of acetate groups by intracellular esterases renders the molecule fluorescent, and the attachment of fluorescein groups to proteins via aminolysis of the succinimidyl ester ensures that the dye is retained within the cell. As cells divide, CFSE is distributed equally between daughter cells (Lyons and Parish

<u>Table 2.2</u> Antibodies used for Phenotyping of Cell Suspensions by Flow Cytometry

Antibody	Conjugate	Isotype	Specificity	Usage	Source
CD11c	R-PE	Mouse IgG1, κ	Monocytes, granulocytes, natural killer cells	10µL into 200µL volume (10^6 cells maximum)	Becton Dickinson
CD13	R-PE	Mouse IgG1, κ	Granulocytic and monocytic cells, mast cells, GM-progenitor cells.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD14	R-PE, FITC	Mouse IgG1, κ	Monocytes and macrophages.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD15	R-PE	Mouse IgM, κ	Granulocytes and monocytes.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD33	R-PE	Mouse IgG1, κ	Monocytes, activated T cells, myeloid progenitors, mast cells.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD34	R-PE	Mouse IgG1, κ	Haematopoietic progenitor cells, vascular endothelium, some tissue fibroblasts.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD36	R-PE	Mouse IgM	Platelets, endothelial cells, monocytes	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD38	R-PE	Mouse IgG1, κ	Thymocytes, activated T cells, plasma cells, monocytes, macrophages, DC, epithelial cells.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD80	FITC	Mouse IgM	Activated B cells, macrophages, DC	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
CD86	R-PE	Mouse IgG1, κ	Monocytes, activated B cells	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
HLA-DR	R-PE	Mouse IgG2a, κ	APC, B cells, monocytes, macrophages, thymic epithelial cells, activated T cells.	10µL into 200µL volume (10^6 cells maximum)	Beckton Dickinson
MAX.1	Supernatant	Mouse IgG	Detects Carboxypeptidase M. Expressed on monocytes and macrophages. Higher expression on macrophages than monocytes.	Cells resuspended in 200µL volume of neat supernatant.	Dr. R. Andreesen, University of Regensburg, Germany
c-fms	Purified	Mouse IgG1	Detects human M-CSF receptor.	1µg/10^6 cells in 400µL volume	R&D Systems

1994) and, using flow cytometry, the number of divisions the cells have undergone can be tracked and correlated with the expression of other proteins.

For CFSE labelling of up to 1.0×10^7 cells, 2μL of 5mM CFSE stock (in DMSO) was diluted in 500μL HBSS/0.1% BSA. This CFSE solution was added to cells that had been suspended in 500μL ice-cold HBSS/0.1% BSA, giving a final concentration of 10μM CFSE. The cell suspension was vortexed thoroughly and incubated at 37°C in a water bath for 10 minutes. Excess CFSE was quenched by adding 3.0mL IMDM/10% FCS and centrifuging for 2 minutes at 815g in a DiaCent-12 mini centrifuge (DiaMed). Cells were washed an additional time with IMDM/10% FCS prior to culture or sorting on a flow cytometer.

Due to a heterogeneous fluorescence profile, CFSE labelled $CD34^+$ cells were sorted over a narrow range of fluorescence intensity (40 channels) (FITC fluorescence) using a FACStar^{PLUS} flow cytometer (Becton Dickinson), according to the method described by (Nordon *et al.* 1997). This technique ensured that division peaks could be easily identified following cell culture.

CFSE division analysis was performed using an Epics®-XL-MCL cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter). To calculate the percentage of starting cells that entered cell division, the percentage of cells in each cell division cycle was determined using a marker tool which was set according to the maximum fluorescence of undivided, non-stimulated controls, and the following formula applied:

$$\textbf{Percentage of starting cells entering division} = 100 - \left(\frac{n_0}{(n_0/2^0) + (n_1/2^1) + (n_2/2^2) + (n_3/2^3) + (n_4/2^4) + \ldots} \right) \times 100$$

where n is the number of cells in a given division cycle, and 0, 1, 2 … denotes the division cycle number (eg n_0 = undivided cells, n_1 = cells in division 1, n_2 = cells in division 2…..).

To calculate the proliferation index, the percentage of cells in each cell division cycle was determined using a marker tool which was set according to the maximum fluorescence of undivided, non-stimulated controls, and the following formula applied:

$$\textbf{Proliferation index} = \left(\frac{n_0 + n_1 + n_2 + n_3 + n_4 + \ldots\ldots}{(n_0/2^0) + (n_1/2^1) + (n_2/2^2) + (n_3/2^3) + (n_4/2^4) + \ldots} \right)$$

where n is the number of cells in a given division cycle, and 0, 1, 2 … denotes the division cycle number (eg n_0 = undivided cells, n_1 = cells in division 1, n_2 = cells in division 2…..).

2.3.7 Preparation of Mononuclear Cells (MNC)

Normal bone marrow (BM) was aspirated into preservative-free, sodium heparin-containing tubes (1,000 units/ml; Fisons Pharmaceuticals, Homebush, NSW, Australia), from the sternum and posterior iliac crest of healthy volunteers following informed consent. Alternatively, peripheral blood (PB) from CML patients was collected in preservative-free, sodium heparin-containing tubes. The Human Ethics Committee, Royal Adelaide Hospital approved the use of BM and PB cells for these studies.

To isolate low-density mononuclear cells (MNC), 40mL of BM or PB was diluted to 100mL with blocking buffer. Forty millilitres of this suspension was layered over 3mL of Ficoll-Hypaque in 15mL round bottom tubes (Falcon), and then centrifuged at 400g for 30 minutes at RT using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge. Interface cells, which comprised of MNC, were transferred to a clean 50mL conical tube using a transfer pipette and washed twice with HHF. White cells were then enumerated using white cell fluid.

2.3.7.1 Isolation of CD34$^+$ Cells: Dynabead Separation

Unless otherwise specified, the isolation of CD34$^+$ cells was performed on MNC from the BM of normal donors or from the PB of CML patients. MNC were washed twice with Isolation buffer (IB) prior to the addition of anti-CD34 Dynabeads (Dynal, Sweden) at a 10:1 ratio of beads:cells in a 5mL polypropylene round bottom tube (Falcon). The beads were washed twice with IB to remove any unbound antibody prior to use, and were resuspended in approximately 200µL of IB. Cells were also resuspended in approximately

200μL of IB. The cells and beads were then combined, and the cell:bead suspension mixed constantly over ice for 3 minutes. The total volume was increased to approximately 4mL with IB, and this suspension was incubated on a rotator for 60 minutes at 4°C.

Cells rosetted by the CD34 Dynabeads were purified by multiple rounds of capture and washing using IB and a cobalt-samarium magnet (magnetic particle concentrator-1 (MPC-1), Dynal, Sweden). Washing involved placing the 5mL polystyrene tube containing the cell suspension on the MPC-1 for 2 minutes, after which time the supernatant (containing $CD34^-$ cells) was aspirated. The cell:bead complex was then resuspended in approximately 4mL IB and returned to the MPC-1. The washing was performed until no free cells remained (approximately 8 washes), this being determined by analysing small samples under a haemocytometer at 10x magnification. Washed cells:beads were then resuspended in 200μL IB.

$CD34^+$ cells were recovered by resuspending the washed cell:bead complexes in DETACHaBEAD™ reagent (Dynal, Sweden) for 1 hour in a 37°C shaking water bath, according to the manufacturers recommendation. The released $CD34^+$ cells were isolated from the magnetic beads by washing the suspension several times using the MPC-1 and IB, as described above, except that the supernatant was aspirated and retained as it contained the $CD34^+$ fraction. Recovered cells were washed once using the MPC-1 to remove any magnetic bead contamination, and a portion of the cell suspension was then labelled with an anti-CD34-PE antibody (as described in section 2.3.6.1) in order to assess purity. In all experiments, this procedure yielded $CD34^+$ populations which were > 90% pure.

2.3.7.2 Isolation of $CD34^+$ Cells: MACS Separation

Unless otherwise specified, the isolation of $CD34^+$ cells was performed on MNC from the BM of normal donors or from the PB of CML patients. MNC were washed twice with MACS $CD34^+$ buffer, and $CD34^+$ progenitor cells were purified using a MACS $CD34^+$ progenitor cell selection isolation kit (Miltenyi Biotech, Germany) according to the manufacturers instructions. Briefly, cells were suspended in 300μL MACS $CD34^+$ buffer per 10^8 cells, and 100μL of both FcR Blocking Reagent and Hapten-Antibody added per 10^8 cells. The suspension was mixed well and incubated for 45 minutes at 4°C. Cells were

washed twice in MACS CD34$^+$ buffer using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge and resuspended in 400µL MACS buffer per 10^8 cells. MACS Anti-Hapten MicroBeads (100µL per 10^8 cells) were added to magnetically label the cells, and the suspension was mixed well and incubated at 4°C for 45 minutes. The cells were washed twice with MACS CD34$^+$ buffer using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge, and then resuspended in 500µL degassed MACS buffer per 10^8 cells (for less than 10^8 cells, a 500µL volume was used).

A MACS LS$^+$ column was placed in a mini-MACS separator magnet and pre-washed with 3mL of degassed MACS CD34$^+$ buffer. The cell suspension was then placed onto the column and the column was washed with cold buffer until no cells were detected in the effluent. Labelled cells within the column were removed by adding 3mL buffer to the column, removing the column from the magnet, and plunging. The eluate was collected in a 10mL polypropylene conical tube (Falcon) and centrifuged for 5 minutes in a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge. The cells were resuspended in 500µL of buffer and reloaded onto a fresh MACS LS$^+$ column that had been pre-washed with 3mL of degassed MACS CD34$^+$ buffer. The column was washed with cold buffer until no cells were detected in the effluent. Labelled cells within the column were removed by adding 3mL buffer to the column, removing the column from the magnet, and plunging. The eluate containing the CD34$^+$ cells was collected in a 10mL polypropylene conical tube and centrifuged for 5 minutes in a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge.

The purity of CD34$^+$ cells following the isolation procedure was calculated by staining with an anti-CD34-PE, as described in section 2.3.6.1.

2.3.8 CFSE CD34$^+$ Cell Assays

2.3.8.1 Establishment of Cultures

CD34$^+$ cells were isolated from the BM of normal donors or PB of CML patients using MACS separation, as described in section 2.3.7.2. CD34$^+$ cells were then labelled with CFSE and sorted to select cells that fell within a range of 40 channels of CFSE fluorescence, as described in section 2.3.6.2.

Sorted cells were resuspended at a concentration of $5.0x10^4$ cells/mL in SDM supplemented with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) (Peprotech). Volumes (200µL) of cell suspension were transferred to wells of a 96 well, flat-bottomed plate, and plates incubated in a humidified chamber at 37°C + 5% CO_2. Control populations not stimulated with growth factors or not stained with CFSE were included in all experiments.

2.3.8.2 Analysis of CFSE CD34$^+$ Cell Cultures

Samples were removed from culture at 24 hour time points over 4 days of culture, and stained with PE-conjugated antibodies detecting CD34, CD38, HLA-DR, CD33, CD13, CD36, CD14, CD11c and CD15, as described in section 2.3.6.1. Samples were then analysed for FITC (CFSE) and PE fluorescence using an Epics®-XL-MCL flow cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter). Controls included cultured cells not stained with CFSE (unstained control for background fluorescence) and CFSE labelled cells not stimulated with growth factors (control used to set fluorescence value for undivided cells).

2.3.9 Haemopoietic Colony Assays

2.3.9.1 Establishment of Colony Assay Cultures

BMMNC or CD34$^+$ cells were assayed for their ability to form colonies on semi-solid agar, based on a modification of a method previously described by Johnson *et al.* (1980). Colony cultures were established in triplicate by plating $5.0x10^4$ BMMNC, or $7.5x10^3$ CD34$^+$ cells, per 35mm cell culture dish (Falcon), in 1.0mL of IMDM supplemented with 0.33% agar, 25% FCS, and 2mm L-glutamine. This was performed by preparing 10mL volumes of cell suspension by combining 5.0mL of 0.66% agar (held at 41°C), 5.0mL of A-IMDM (held at 37°C) and a volume of cell suspension corresponding to either $5.0x10^5$ BMMNC, or $7.5x10^4$ CD34$^+$ cells. The solution was swirled quickly, and 1.0mL aliquots dispensed onto pre-prepared 35mm cell culture dishes (see below), ensuring that no air bubbles formed. The cultures were left to air dry at room temperature for approximately 10 minutes, before being transferred to a humidified chamber and incubated at 37°C + 5% CO_2 for a period of 14 days.

Pre-preparation of 35mm cell culture dishes involved dispensing growth factors and/or drugs onto the centre of the dishes prior to the addition of the cell suspension. Growth factors (50μL per plate) used to stimulate colony growth included M-CSF (final concentration 25ng/mL, Peprotech), GM-CSF (final concentration 10ng/mL, Peprotech) PDGF (final concentration 250ng/mL, Peprotech), or a combination of 2 growth factors (GM-CSF (final concentration 10ng/mL) and M-CSF (final concentration 25ng/mL)), 4 growth factors (IL-3, IL-6, G-CSF, GM-CSF, each at a final concentration of 10ng/mL) or 5 growth factors (IL-3, IL-6, G-CSF, GM-CSF, SCF, each at a final concentration of 10ng/mL) (Peprotech). Imatinib (Novartis, Basel, Switzerland) was also added to cultures in triplicate, to a final concentration ranging from 0.3μM to 50.0μM. Control cultures with no added growth factors or no added imatinib were also included in all experiments. The effect of blocking antibodies on colony growth was also examined in several experiments, and these antibodies included α-c-fms (2-4A5, Santa Cruz Biotechnology Inc) (1μg/mL) and α-c-kit (Sigma) (1μg/mL).

2.3.9.2 Fixation of Colony Assay Cultures

After 14 days in culture, colony cultures were fixed by the addition of 1.0mL of 3% glutaraldehyde, and left for 1-2 days at 4°C. Fixed agar cultures were then rinsed with PBS and transferred to glass slides. This was performed by floating the agar plugs in PBS and covering the top of the culture dish with a glass slide (75mm x 50mm). The slide/dish was then flipped 180°, and the surface tension between the slide and dish broken by gently inserting a scalpel blade. The culture dish was then removed, leaving the agar plug on the glass slide. Agar plugs were dried down by covering the slides with moistened nitrocellulose paper, and leaving to dry for several hours. The slides were then dipped quickly into Milli-Q® water, and the nitrocellulose paper gently removed. Slides were then ready for staining.

2.3.9.3 Tri-staining of Colony Assay Cultures

Colony assay slides were sequentially stained for naphthol acetate esterase (Lojda 1979) and chloroacetate esterase (Kubota *et al.* 1980), and stained with luxol fast blue dye (BDH) (Metcalf 1984), to identify monocyte/macrophage, neutrophil and eosinophil colonies

respectively. Slides were then enumerated for each of the colony types, with all colonies being scored according to standard criteria ($\geq$50 cells per colony).

2.3.9.3.1 Naphthol acetate esterase staining:

Twenty-five millilitres (25mL) of 2-ethoxyethanol was combined with 25 drops of α-naphthol butyrate in a 1.0L beaker. To this, 1.0L of 0.067M phosphate buffer pH 6.3 and 0.5g Fast Garnet GBC was added. The solution was mixed thoroughly then filtered quickly using Whatman paper. Slides were stained for a period of 1 hour at RT, and then washed thoroughly with tap water.

2.3.9.3.2 Chloroacetate esterase staining:

Fifty millilitres (50mL) of N,N-dimethyl formamide was combined with 0.1g of Naphthol AS-D chloroacetate in a 1.0L beaker. To this, 0.5g Fast Blue BB and 1.0L 0.067M phosphate buffer pH 7.4 was added. The solution was mixed thoroughly then filtered quickly using Whatman paper. Slides were stained for a period of 1 hour at RT, and then washed thoroughly with tap water.

2.3.9.3.3 Luxol fast blue dye:

Luxol fast blue (1.5g) was dissolved in 1.0L of urea-saturated ethanol. The solution was filtered using Whatman paper, and slides stained for a period of 2 hours at RT. Slides were washed thoroughly with tap water and mounted in DePeX mounting medium (Gurr®, BDH).

2.3.10 CD34$^+$ Liquid Culture Assays

CD34$^+$ cells were isolated from BMMNC using Dynabead magnetic separation, as described in section 2.3.7.1, and incubated in serum-deprived medium (SDM) at a final concentration of 5.0x10^3 cells/mL. IL-3, IL-6, G-CSF and GM-CSF were added to a final concentration of 10ng/mL (Peprotech). Cell culture flasks (25cm^2) (Cellstar®, Greiner bio-one, Germany) were seeded with a 5.0mL volume of cell suspension, and imatinib added to duplicate flasks at a final concentration ranging from 0.3µM to 30.0µM. Cells were re-fed with growth factors at a final concentration of 10ng/mL after 7 days, and supplemented with 15.0mL additional medium after 14 days, according to a modification

of the method by Haylock *et al.* (1992). Cells remained in culture at 37°C + 5% CO_2 for a period of 21 days, and viability was assessed at weekly intervals using trypan blue exclusion.

After 21 days, cultures were assayed for cell count and viability using trypan blue exclusion. Cytospin preparations were examined following Jenner-Giemsa staining and Esterase staining, and cells were phenotyped by flow cytometry using directly conjugated antibodies against CD14 (FITC), CD34 (PE), CD11c (PE) and CD33 (PE) (Becton Dickinson, CA), as described in section 2.3.6.1.

2.3.11 Monocyte Cultures

2.3.11.1 Isolation of Monocytes

Buffy coats from normal healthy volunteers were obtained from the Australian Red Cross Blood Service, Adelaide, with ethical approval. Cells were diluted 1:4 with HBSS, and 40mL volumes of this cell suspension were layered over 10mL of Ficoll-Hypaque in 50mL conical tubes. Suspensions were centrifuged at 400g for 30 minutes, and interface cells constituting mononuclear cells (MNC) were removed using a transfer pipette. Cells were washed once in HHF prior to the next step.

Mononuclear cells were further enriched for monocytes using a Percoll continuous gradient. All MNC isolated from the buffy coat were resuspended in 3.5mL RPMI (JRH Biosciences) in a 15mL round bottom tube (Falcon), and combined with 6.7mL 0.99g/mL Percoll solution. The suspension was mixed well and centrifuged in a fixed angle rotor (Beckman Avanti-J-25I centrifuge with JA-25.50 rotor) at 400g for 25 minutes at RT. The surface layer of cells was removed using a transfer pipette, and routinely comprised of 65-80% monocytes.

Monocytes were further purified using a MACS® negative selection monocyte kit (Miltenyi Biotech, Germany) according to the manufacturers instructions. Briefly, cells were suspended in 60µL MACS monocyte buffer per 10^7 cells, and 20µL of both FcR Blocking Reagent and Hapten-Antibody Cocktail added per 10^7 cells. The suspension was mixed well and incubated for 5 minutes at 6-12°C. Cells were washed twice in MACS

monocyte buffer using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge and resuspended in 60μL MACS monocyte buffer per 10^7 cells. FcR Blocking Reagent (20μL per 10^7 cells) and MACS Anti-Hapten MicroBeads (20μL per 10^7 cells) were added to magnetically label the cells, and the suspension was mixed well and incubated at 6-12°C for 15 minutes. The cells were washed once with MACS monocyte buffer using a Heraeus multifuge $3_{S\text{-}R}$ bench top centrifuge, and then resuspended in 500μL degassed MACS monocyte buffer per 10^8 cells (for less cells, still used 500μL).

A MACS LS^+ column was placed in a mini-MACS separator magnet and washed with 3mL of degassed MACS monocyte buffer. The cell suspension was then placed onto the column, and the effluent collected as the negative fraction representing monocytes. The column was rinsed with 12mL of buffer to ensure a high yield of monocytes, and the column then discarded. The purity of monocytes following the isolation procedure was calculated by staining with an anti-CD14-PE (Beckton Dickinson), as described in section 2.3.6.1, and was routinely greater than 95%.

2.3.11.2 Establishment of Monocyte Cultures

Monocytes were resuspended in serum deprived medium (SDM) to a final concentration of $1.0x10^5$ cells/mL, and supplemented with rh-M-CSF (40ng/mL final concentration) or rh-GM-CSF (20ng/mL final concentration) (Peprotech). One millilitre (1.0mL) volumes of cell suspension were transferred to wells of a 24 well cell culture plate, and imatinib added to duplicate wells at a concentration ranging from 0.3μM to 30.0μM. Cultures were incubated in a humidified chamber at 37°C + 5% CO_2.

After a period of 7 days, cultures were examined for changes in cell morphology (increase in cell size, formation of pseudopodia) and photos were taken using an Olympus DP11 digital camera. Cells were detached from culture plates by removing the supernatant, washing the wells twice with PBS, and incubating the cells in Cell Dissociation Buffer (Gibco) for 20 minutes at 37°C. The cells were then detached by vigorous pipetting.

The maturity of cells was examined by flow cytometric analysis of cell suspensions following staining with antibodies directed against CD14 (PE), HLA-DR (PE), CD11c

(PE) and Carboxypeptidase M (MAX.1, mouse IgG supernatant: generously donated by R. Andreesen, Department of Haematology and Oncology, University of Regensburg, Germany), as described in section 2.3.6.1.

Chamber slide (Lab-Tek®, Nalge Nunc International, USA) cultures were also established using the method described above, except that 300μL volumes of cell suspension were used per well. At the conclusion of the culture period, the supernatant was aspirated and the cells fixed for 15 minutes in 300μL of 3% glutaraldehyde. Cells were then examined following staining for naphthol acetate esterase, as described in section 2.3.9.3.

2.3.12 Monocyte/Macrophage Functional Assays

2.3.12.1 Morphologic Response to Activation with Lipopolysaccharide

Monocyte cultures were established in chamber slides, as described in section 2.3.11.2. After 5 days in culture, LPS (Sigma) was added to a final concentration of 1μg/mL. After a further 2 days in culture, the supernatant was aspirated and the cells fixed for 15 minutes in 300μL of 3% glutaraldehyde. Cells were then examined following staining for naphthol acetate esterase, as described in section 2.3.9.3.

2.3.12.2 ELISAs to Measure IL-6 & TNF-α Production

ELISA kits designed to specifically measure IL-6 and TNF-α were purchased from BenderMed Systems (Vienna, Austria), and ELISAs performed according to the manufacturer's instructions. Briefly, 96 well, flat bottomed Maxisorp Nunc-Immuno™ plates (Nalge Nunc International, Denmark) were incubated overnight at 4°C in coating antibody (2.5μg/mL in PBS, 100μL antibody solution per well). Plates were washed once with PBS/0.5% tween-20, and then blocked by adding 200μL IL-3/IL-6 assay buffer to each well. Blocking was performed for 2 hours at RT, and was followed by washing the plates twice with PBS/0.5% tween-20.

While plates were blocking, samples were diluted appropriately (1/50 for TNFα, 1/400 for IL-6) in IMDM/1% BSA and kept on ice until used. Standards were also prepared by diluting 20μL 1ng/mL IL-6 standard in 180μL IMDM/1% BSA, and/or 10μL 10ng/mL

standard in 190µL IMDM/1% BSA. Prepared standards (200µL) were dispensed into the first wells, and then serially diluted across 6 wells containing 100µL IMDM/1% BSA. Samples (100µL/well) were loaded onto plates in duplicate, and 50µL of specific biotin conjugate (1/500 dilution in IL-3/IL-6 assay buffer) added to all wells. Prepared plates were then incubated at RT for 2 hours.

Plates were washed 4 times using PBS/0.5% tween-20, and 100µl of diluted Streptavidin-horseradish peroxidase conjugate added to each well (1/5000 dilution in IL-3/IL-6 assay buffer for IL-6, and 1/10000 dilution in IL-3/IL-6 assay buffer for TNF-α). Plates were incubated at RT for 1 hour, and washed 4 times with PBS/0.5% tween-20. TMB substrate (100µL/well) was added to each well, and plates incubated for 10-20 minutes at RT in the dark. The reaction was stopped by adding 100µL 4N (2M) sulphuric acid to each well, and the plates were read immediately on an ELISA plate reader set at 450nm.

2.3.12.3 ELISA to Measure M-CSF Production

A Human M-CSF DuoSet® ELISA Development System kits was purchased from R&D Systems, and ELISAs performed according to the manufacturer's instructions. Briefly, 96 well flat bottomed Maxisorp Nunc-Immuno™ plates (Nalge Nunc International, Denmark) were incubated overnight at 4°C in coating antibody (2.0µg/mL in PBS, 100µL antibody solution per well). Plates were washed three times with PBS/0.05% tween-20 and blocked by adding 300µL M-CSF assay buffer to each well. Blocking was performed for 1 hour at RT, and was followed by washing the plates 3 times with PBS/0.05% tween-20.

Standards were prepared by serially diluting a high standard of 1000pg/mL in M-CSF assay buffer, and 100µL volumes transferred to a 96 well plate. Samples (100µL/well, neat) were loaded onto plates in duplicate, and incubated for 2 hours at RT.

Plates were washed 3 times using PBS/0.05% tween-20, and 100µl of detection antibody (50ng/m in M-CSF assay buffer) added to each well. Plates were incubated at RT for 2 hours, and washed 3 times with PBS/0.05% tween-20. Streptavidin-HRP (100µL of working dilution in M-CSF assay buffer) was added to each well, and the plates incubated

for 20 minutes at RT. Plates were washed 3 times with PBS/0.05% tween-20, and 100µL TMB substrate added to each well. Plates were incubated for 20 minutes at RT in the dark, and the reaction stopped by adding 50µL of 2N (1M) sulphuric acid to each well. Plates were read immediately on an ELISA plate reader set at 450nm.

2.3.12.4 Antigen Uptake Assay: Zymosan Bioparticles®

Monocyte cultures (1.0mL) were established in 24 well plates as described, and after a period of 7 days, $2x10^6$ fluorescein conjugated zymosan (FITC-zymosan) or opsonised FITC-zymosan Bioparticles® (Molecular Probes) were added to each 1.0mL of culture (20:1 Bioparticles®:cells). Plates were incubated at 37°C + 5% CO_2 for 1 hour, and the wells washed 3 times with 1.0mL PBS. Cultures were then extensively trypsinised for 1 hour to remove non-phagocytosed zymosan Bioparticles®.

Cultured monocytes were detached from plates by incubating plates with 0.5mL cell dissociation buffer (Gibco) at 37°C for 20 minutes, followed by gentle pipetting. Cells were then analysed for fluorescence using an Epics®-XL-MCL flow cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter).

Monocyte cultures established in chamber slides were also treated with FITC-zymosan or opsonised FITC-zymosan (20:1 Bioparticles®:cells) for 1 hour at 37°C, washed three times with PBS, and extensively trypsinised. Slides were then fixed and stained for naphthol acetate esterase, and analysed using a Bio-RAD radiance 2100 confocal microscope (Bio-RAD microscience Ltd, UK).

2.3.12.5 Mixed Lymphocyte Reaction (MLR)

Responder cells were isolated from buffy coats from normal healthy volunteers using a combination of a Ficoll-Hypaque gradient to isolate MNC, and a nylon wool column to enrich the population for T cells.

Briefly, buffy coats from normal healthy volunteers were obtained from the Australian Red Cross Blood Service, Adelaide, with ethical approval. Cells were diluted 1:4 with HBSS, and 40mL volumes of this cell suspension were layered over 10mL of Ficoll-Hypaque in

50mL conical tubes. Suspensions were centrifuged at 400g for 30 minutes and interface cells constituting mononuclear cells (MNC) were removed using a transfer pipette. Cells were washed once in HHF, and then resuspended at $1.0x10^8$/mL in warm RPMI/10% FCS.

Nylon wool columns were prepared by repeatedly teasing 0.6g of nylon wool until a fine network of fibres was obtained, then rolling/folding the wool and placing in a 10mL syringe barrel. After autoclaving, a disposable three-way-stopcock (Terumo) was placed on the end of the syringe, and warm RPMI/10% FCS added to the syringe drop wise, until the RPMI/10% FCS exited the syringe. The stopcock was then closed, and warm RPMI/10% FCS added until the nylon wool was well covered with medium. Air bubbles trapped within the nylon wool were released by gently teasing the nylon wool with a Pasteur pipette, and the nylon wool was compressed until it was approximately 2cm thick. The stop-cock was opened until the barrel was almost empty, the ends of the barrel sealed with parafilm, and the column incubated for 45 minutes at 37°C + 5% CO_2.

After equilibration of the column, the stopcock was opened and the cells added drop-wise to allow even distribution. The column was then washed with 2.0mL medium, the barrel sealed with parafilm, and the column incubated for 45 minutes at 37°C + 5% CO_2.

T-cell enriched populations ("nylon wool-enriched cells") were released from the column by washing with approximately 12mL of warm RPMI/10% FCS. Cells were then stained with 10μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (see section 2.3.6.2) and resuspended at $1.0x10^6$ cells/mL in RPMI/10% FCS.

Monocyte cultures established in 24 well plates were incubated for a period of 5 days, then washed three times using RPMI/10% FCS to remove imatinib. CFSE labelled "nylon wool-enriched cells" were then added to washed monocyte cultures ($1.0x10^6$ cells/well), and returned to the incubator for a further 5 days. The non-adherent cells were harvested from the culture and examined for CFSE fluorescence using an Epics®-XL-MCL flow cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter). Gates were drawn to select blasting cells (cells with increased forward and side scatter compared

to control), and gated cells were analysed for their CFSE fluorescence profile. The equation used to calculate the percentage of undivided cells is displayed in section 2.3.6.2

2.3.13 Proliferation Assay: Flow Beads

Flow-Check™ Fluorospheres (Beckman Coulter) were washed twice in HBSS/0.1% BSA/0.1% NaN_3 and resuspended at $1.0x10^6$ beads/mL in HBSS/0.1% BSA/0.1% NaN_3.

FDC-cfms cells (see section 2.5.5) were resuspended at $5.0x10^4$/mL in $DMEM^+$, and supplemented with either murine IL-3 (1:2000) or rhM-CSF (60ng/mL). Volumes (200µL) were transferred to wells of a 96 well plate, and imatinib added to a final concentration of 0.5µM, 1.0µM, 2.5µM and 5.0µM, in triplicate. Triplicate plates were incubated at 37°C + 5% CO_2, and at 12, 24 and 48 hour time points, wells were harvested and transferred to 5.0mL tubes (Falcon). Cells were fixed in a final volume of 400µL FACS fixative, and a fixed volume of known density flow beads were then added to each tube. Tubes were vortexed thoroughly, and cells analysed using an Epics®-XL-MCL flow cytometer and Cytomics™ RXP Analysis Version 1.0 Software (Beckman Coulter). Gates were drawn on FS v SS plots around regions that corresponded to beads or cells, and percentages falling within these gates were used to calculate the corresponding cell densities, using the formula:

$$\text{Total no. cells} = \left(\frac{\text{no. events in cell gate}}{\text{no. events in bead gate}}\right) \text{ x } \left(\frac{\text{bead volume}}{\text{cell volume}}\right) \text{ x bead density x cell volume}$$

2.4 Molecular Biology Reagents

2.4.1 Loading Buffer, 2x Denaturing

Tris-HCl (0.5M, pH 6.8)	4.0mL
SDS (10%)	6.4mL
Glycerol (100%)	3.2mL
β-mercaptoethanol	1.6mL
Bromophenol Blue (0.05%)	0.8mL

Reagents were combined, and 1.0mL aliquots stored at -20°C.

2.4.2 Loading Buffer, 2x Non-Reducing

Tris-HCl (0.5M, pH 6.8)	4.0mL
SDS (10%)	6.4mL
Glycerol (100%)	3.2mL
Bromophenol Blue (0.05%)	0.8mL
Milli-Q® water	1.6mL

Reagents were combined, and 1.0mL aliquots stored at -20°C.

2.4.3 Luria Broth(LB)

Bacto tryptone (BD)	10.0g
Bacto yeast extract (Difco, BD)	5.0g
NaCl	10.0g
Milli-Q® water	make up to 1L

The Bacto tryptone, Bacto yeast extract and NaCl were dissolved in Milli-Q® water using a magnetic stirrer. The solution was sterilised by autoclaving on a fluid cycle, and stored at room temperature.

2.4.4 LB Agar

Luria Broth	500mL
Bacto agar (Difco, BD)	7.5g

The Bacto agar was dissolved in luria broth using a magnetic stirrer. The solution was autoclaved on a fluid cycle, and cooled with gentle mixing using a magnetic stirrer to prevent solidification. Ampicillin (100µg/mL) was added to the LB agar when cool enough to handle, and plates poured using 35mm bacterial grade petri dishes (Techno-Plas). LB agar plates were stored at 4°C.

2.4.5 Lysis Buffer

1% NP-40 in TSE	17.6mL
NaF (0.5M)	200µL
NaPPi (0.1M)	1.0mL
Sodium vanadate (0.5M)	200µL
Complete protease inhibitors*	400µL
Leupeptin (1mg/mL)	200µL
Aprotinin (1mg/ML)	200µL
PMSF (0.1M)	200µL

Lysis buffer was prepared freshly on the day of use and kept at 4°C. PMSF was added just prior to lysing.

* For the complete protease inhibitors (Roche Diagnostics), 1 cocktail tablet was dissolved in 1.0mL Milli-Q® water. The solution was stored at -20°C, and used within 1 week of preparation.

2.4.6 Membrane Blocking Solution

Membrane blocking agent	2.5g
1x TBS	100mL

The membrane blocking agent (Amersham) was dissolved in 1x TBS with gentle agitation. The solution was prepared fresh on the day of use.

2.4.7 5% Polyacrylamide Gel (Stacking Gel)

Milli-Q® water	1.8mL
Acryl (40%)	380µL
Tris (0.5M)	760µL
SDS (10%)	30µL
APS (10%)	30µL
Temed	3µL

Reagents were all combined just prior to pouring of the gel.

2.4.8 8% Polyacrylamide Gel (Resolving Gel)

Milli-Q® water	2.6mL
Acrylamide (40%)	1.0mL
Tris (1.5M)	1.25mL
SDS (10%)	50µL
APS (10%)	50µL
Temed	3µL

Reagents were all combined just prior to pouring of the gel.

2.4.9 SDS-PAGE Electrode Buffer (25mM Tris/ 192mM Glycine/ 0.1% SDS)

Tris	3.026g
Glycine	14.413g
SDS	1.0g
Milli-Q® water	make volume up to 1.0L

Tris, glycine and SDS were dissolved in Milli-Q® water with the aid of a magnetic stirrer. The pH of the solution should be 8.3 if it is ensured that all reagents are accurately weighed.

2.4.10 TBS Stock (10x)

Tris	24.2g
NaCl	87.6g
Milli-Q® water	make up to 1.0L

Tris and NaCl were dissolved in approximately 800mL of Milli-Q® water, and the pH adjusted to pH 7.5 using concentrated HCl. The volume was then made up to 1.0L with Milli-Q® water.

2.4.11 1x TBS

10x TBS stock	100mL
Milli-Q® water	900mL

The 10x TBS stock was diluted 1/10 with Milli-Q® water.

2.4.12 TBS-T (1x TBS / 0.1% Tween)

10x TBS stock	100mL
Tween-20	1.0mL
Milli-Q® water	899mL

The 10x TBS stock was combined with Tween-20, and the solution diluted 1/10 with Milli-Q® water. The solution was stored at 4°C.

2.4.13 TE Buffer (10x)

Tris	2.422g
EDTA	0.744g
Milli-Q® water	make up to 200mL

Tris and EDTA were dissolved in approximately 150mL of Milli-Q® water, and the pH adjusted to pH 7.4 using concentrated HCl. The volume was made up to 200mL with Milli-Q® water.

2.4.14 Transfer Buffer (25mM tris, 192mM glycine, 20% methanol)

Glycine	14.413g
Tris	3.026g
Methanol	200mL
Milli-Q® water	make up to 1L

Glycine and tris were dissolved in Milli-Q® water with the aid of a magnetic stirrer. Methanol was then added, and the volume made up to 1L with Milli-Q® water. The transfer buffer was stored at 4°C.

2.4.15 1.5M Tris (pH 8.8)

Tris	18.165g
Milli-Q® water	make up to 100mL

Tris was dissolved in approximately 80mL of Milli-Q® water, and the pH adjusted to pH 8.8 using concentrated HCl. The volume was then made up to 100mL with Milli-Q® water.

2.4.16 0.5M Tris (pH 6.8)

Tris	6.052g
Milli-Q® water	make up to 100mL

Tris was dissolved in approximately 80mL of Milli-Q® water, and the pH adjusted to pH 6.3 using concentrated HCl. The volume was then made up to 100mL with Milli-Q® water.

2.4.17 TSE Buffer (50mM tris, 100mM NaCl, 1mM EDTA, pH 8.0)

Tris	6.052g
NaCl	8.760g
EDTA	0.372g

Tris, NaCl and EDTA were dissolved in approximately 950mL of Milli-Q® water, and the pH adjusted to pH 8.0. The volume was then made up to 1.0L with Milli-Q® water.

2.5 Molecular Biology Techniques

2.5.1 Transformation of Competent Cells

An MSCV-CSF1Rwt-IRES GFP plasmid containing a human wild-type c-fms insert was obtained from C. Sherr, St Jude Children's Research Hospital, Memphis, Tennessee, USA. The DNA was eluted from the filter paper by cutting the filter paper into small fragments, and soaking in 100µL TE buffer for 2 hours at room temperature in a 1.5mL Eppendorf.

The plasmid was transformed into competent cells by combining 2µL of the eluted DNA with 50µL of an XL1-blue strain of competent *Escherichia coli* in a 1.5mL Eppendorf. The tube was incubated on ice for 20 minutes, and then heat-shocked at 42°C for 90 seconds. The tube was returned to ice for 2 minutes, and 1.0mL of LB added. The tube was incubated on a shaker at 37°C for 45 minutes to allow recovery of the cells, and 200µL of this suspension then streaked on an LB agar plate that was supplemented with 100µg/mL ampicillin. Plates were incubated upside down overnight at 37°C

Cells that were successfully transformed with MSCV-CSF1Rwt-IRES GFP plasmid would contain an ampicillin resistance gene, and therefore be able to grow on LB agar supplemented with ampicillin. Transformed colonies were plucked and dipped into a 5mL tube containing 3mL LB supplemented with 100µg/mL ampicillin, and incubated overnight at 37°C on a shaker. These cultures were then used for mini-prep isolation of plasmid DNA.

2.5.2 Purification of Plasmid DNA from Bacterial Cultures

2.5.2.1 Small Scale Plasmid DNA Extraction (Mini-Prep Protocol)

Tubes containing bacterial cultures were mixed well, and 1.5mL volumes transferred to 1.5mL Eppendorf tubes. Suspensions were centrifuged at 13,000rpm for 3 minutes in Heraeus Biofuge pico centrifuge, and the supernatant aspirated. Cold resuspension buffer (100µL) (Qiagen Buffer P1 + RNaseI) was added to each tube, and the bacteria resuspended by pipetting. Lysis buffer (100µL) (Qiagen Buffer P2) was added, and the tube inverted several times to mix gently, before adding 100µL cold neutralisation buffer (Qiagen Buffer P3). Tubes were inverted to mix, then centrifuged at 13,000rpm for 15 minutes.

The supernatant from each tube was harvested and transferred to a clean 1.5mL Eppendorf that contained 30µL 3M sodium acetate, pH 4.8. Ethanol (600µL of 100% solution) was added to each tube, the contents mixed thoroughly, and the tubes centrifuged at 13,000rpm for 30 minutes at 4°C. The supernatant was then aspirated, and the DNA pellets washed

with 1.0mL 80% ethanol. The ethanol was aspirated and DNA pellets air-dried before resuspending in Milli-Q® water. DNA was stored at -20°C.

2.5.2.2 Medium Scale Plasmid DNA Extraction (Midi-Prep Protocol)

One hundred millilitre (100mL) bacterial cultures were set up in conical flasks in LB medium containing 100µg/mL ampicillin and 200µL of the 2mL single colony cultures, and incubated at 37°C overnight with shaking.

The next day, flasks were placed on ice, and the cultures transferred to 250mL centrifuge bottles (Nalgene) and centrifuged at 6000g for 15 minutes at 4°C to harvest the bacteria (Beckman Avanti-J-25I centrifuge with JA-10 rotor). The supernatant was decanted, and excess liquid removed using a sterile Pasteur pipette. Cold resuspension buffer (4.0mL) (Qiagen Buffer P1 + RNaseI) was added to each tube, and the bacteria resuspended by pipetting. Bacterial suspensions were then transferred to a 50mL conical tube (Falcon), 4.0mL lysis buffer (Qiagen Buffer P2) added, and the tube inverted several times to mix gently before adding 4.0mL cold neutralisation buffer (Qiagen Buffer P3).

Tubes were inverted to mix, and the solution transferred to a Qiafilter cartridge (Qiagen) with screw-cap ends attached. The solution was incubated in the Qiafilter for 10 minutes at room temperature, the screw-cap ends removed, and a plunger inserted and depressed to transfer the DNA lysate into a Qiagen Tip column that had been equilibrated with 4.0mL Equilibration buffer (QBT, Qiagen). The lysate entered the resin in the Qiagen Tip column by gravity flow, and was washed twice with 10mL wash buffer (Buffer QC, Qiagen).

Once the Qiagen Tip columns emptied, DNA was eluted from the resin with 5.0mL elution buffer (Buffer QF, Qiagen) into Oak Ridge tubes, and precipitated by adding 3.5mL 100% isopropanol. Tubes were centrifuged for 30 minutes at 15000g in a centrifuge that had been pre-cooled to 4°C (Beckman Avanti-J-25I centrifuge with JA-25.50 rotor).

After centrifugation, the supernatant was aspirated, the DNA pellets resuspended in 400µL TE buffer, pH 7.4, and the solution transferred to 1.5mL Eppendorf tubes. The DNA was precipitated by adding 25µL 3M sodium acetate, pH 4.8, and 1mL 100% ethanol, and

incubating at -20°C for 1-2 hours. Tubes were then centrifuged at 13,000rpm for 30 minutes in an Eppendorf 541SR centrifuge that had been pre-cooled to 4°C. The supernatant was aspirated and DNA pellets washed with 1mL 80% ethanol, ensuring that the pellet was not dislodged. The supernatant was then aspirated and the DNA pellets air-dried and resuspended in 100µL Milli-Q® water.

2.5.3 Manipulation of DNA Products

2.5.3.1 Quantitation of DNA

The concentration of DNA in solution was determined by spectrophotometry A_{260nm}1=50µg/mL (1cm light path).

2.5.3.2 Electrophoresis of DNA

DNA was separated by electrophoresis on 1% agarose (DNA grade, Progen) gels. Gels were loaded into a horizontal electrophoresis tank containing 1x TAE, and DNA samples (5µL) were combined with loading buffer (2µL) (MBI Fermentas) and loaded into the wells. After electrophoresis at 100V, 70-80mA until the bromophenol blue front was three quarters along the gel, gels were stained with ethidium bromide (2µg/mL in water) for 10 minutes. Gels were rinsed quickly with water, and DNA bands visualised on a short-wave UV transilluminator (254nm) and photographed using a Polaroid 600SE camera.

2.5.3.3 Restriction Digest to Check Presence of Insert in Plasmid DNA

Restriction enzyme digests of DNA were performed by digesting 100ng of DNA with 1µL of restriction enzyme in the presence of 1x digestion buffer in a total reaction volume of 10µL. The reaction was carried out in a 37°C heat block for 1-2 hours.

2.5.3.4 Size Determination of DNA Fragments

The size of DNA fragments was calculated by comparing their relative mobilities with those of a DNA standard (SPP1).

2.5.4 Retroviral Transfection of the Ecotropic Packaging Cell Line, ψ_2

FuGENE (3µL) (Roche) was added to 150µL DMEM, and incubated at room temperature for 5 minutes. Plasmid DNA (1µg in 1µL) was then added, and the solution mixed gently

and incubated for 20 minutes at room temperature. Meanwhile, the ecotropic retroviral packaging cell line, ψ_2, was suspended at 2.0x10^5/mL in DMEM$^+$, and 2.0mL volumes transferred to wells of a 6 well plate (Greiner). Once the plasmid DNA/FuGENE had incubated for the required time, the plasmid DNA/FuGENE solution (154µL) was added to the ψ_2 cells dropwise, and swirled to mix thoroughly. Plates were incubated for 24 hours at 37°C + 5% CO_2.

After 24 hours, the medium was aspirated from the wells, and the wells washed with 2.0mL PBS. Trypsin (750µL) was added to each well, and the plates incubated at 37°C + 5% CO_2 for 2 minutes until the cells detached from the plate. Cells were resuspended in 10.0mL DMEM$^+$, transferred to a 75cm^2 tissue culture flask and incubated at 37°C + 5% CO_2. Once cells were approximately 80% confluent, they were detached from the flask using trypsin, and sorted on a FACStar^{PLUS} flow cytometer (Becton Dickinson), collecting cells that expressed green fluorescence protein (GFP).

Sorted GFP$^+$ cells were resuspended in 5.0mL DMEM$^+$, and transferred to a 25cm^2 tissue culture flask (Greiner). Cells were expanded in number until there were sufficient cells to perform retroviral infection.

2.5.5 Retroviral Infection of the Suspension Cell Line, FDC-P1, by Co-cultivation

The murine factor-dependent cell line, FDC-P1, was infected by co-cultivation with virus-producing ψ_2 cells. Briefly, 75cm^2 tissue culture flasks (Greiner) were seeded with 3x10^6 irradiated (30Gy) MSCV-CSF1Rwt-IRES GFP transfected ψ_2 cells in 10.0mL DMEM$^+$, and placed in a 37°C + 5% CO_2 incubator for 3 hours. FDC-P1 cells were resuspended at 1.5x10^5 cells/mL in DMEM$^+$ supplemented with 1/500 murine GM-CSF and 8µg/mL polybrene (4µg/mL final concentration), and a 10.0mL volume of this cell suspension was co-cultivated with the ψ_2 cells for 48 hours. Following this, the FDC-P1 cells were harvested by vigorous agitation of the flask, and infected cells were resuspended at 5x10^4/mL and selected in DMEM$^+$ supplemented with 80ng/mL rhM-CSF. Four to five days following the selection, 95% of the cells were expressing the c-fms protein, and were maintained in DMEM$^+$ supplemented with either 60ng/mL rhM-CSF or 1/2000 murine IL-3.

2.5.6 Immunoprecipitation of c-fms Protein

FDC-cfms or FDC-P1 cells were washed three times and incubated for 1 hour at $1x10^6$ cell/mL in serum free medium (DMEM) at 37°C. Following starvation, cells were recounted and resuspended at $1.5x10^7$/mL in DMEM that had been pre-warmed to 37°C, and 1.0mL volumes transferred to 1.5mL Eppendorfs. Eppendorf tubes were placed in a heat block set at 37°C, and stimulated with 60ng/mL rhM-CSF for 2 minutes. Cells were pelleted by pulsing to 13,000rpm in a Heraeus Biofuge pico, and the supernatant aspirated. Cells were then resuspended thoroughly in 1.0mL ice-cold lysis buffer, and tubes incubated for 30 minutes on ice.

Tubes were centrifuged at 13,000rpm for 30 minutes in an Eppendorf 541SR centrifuge pre-cooled to 4°C, and the supernatant transferred to a clean 1.5mL Eppendorf tube that contained 30µL pre-washed protein G Sepharose (Amersham). Anti-c-fms antibody (2.5µg) (2-4A5, Santa Cruz Biotechnology Inc) or an isotype control were added to appropriate tubes, and the tubes incubated on a rotator for 2 hours at 4°C.

The Sepharose-antibody-protein complex was washed five times with lysis buffer, by centrifuging at 13,000rpm for 1 minute in an Eppendorf 541SR centrifuge pre-cooled to 4°C, and aspirating the supernatant. Equivalent amounts of protein as determined using a Micro BCA™ Protein Assay Reagent (Pierce) were used in each IP, with this assay being performed according to the manufacturer's instructions.

2.5.7 Preparation of Samples for SDS-PAGE

Immunoprecipitates were resuspended in 30µL of 2x non-reduced loading buffer for α-c-fms Western blots and 30µL of 2x reduced loading buffer for α-phosphotyrosine Western blots, in 1.5mL Eppendorf tubes. Samples were then boiled in water for 2 minutes and loaded onto an SDS-PAGE gel with an appropriate protein ladder.

2.5.8 SDS-PAGE Gel Preparation

Glass plates, alumina backing, spacers and combs were cleaned thoroughly with 70% ethanol. The apparatus was assembled and placed into a Hoefer Mighty Small Dual Gel

Caster, and the resolving gel poured until the level sat approximately 1cm below the comb. The gel was then covered with Milli-Q® water to prevent oxidation and left to set for 20 minutes.

When the gel was set, the water layer was poured out, and the excess water removed by blotting with filter paper. The stacking gel was then prepared, poured until it reached the height of the alumina backing, and left to set for 20 minutes.

2.5.9 Loading & Running of SDS-PAGE Gel

The gel apparatus was removed from the gel caster, and placed into a running tank. SDS-PAGE electrode buffer was poured into the space behind the gel plates until the cavity was full, and also poured to a depth of approximately 1 cm in the base of the tank. The comb was removed, and samples and marker loaded. The gel was run at 15mA per gel until samples moved out of the stacking gel, and the current then increased to 20mA per gel.

2.5.10 Transfer of Protein to PVDF using a Semi-Dry Blotting Apparatus

PVDF membrane (Amersham) and Whatman filter paper were cut to an appropriate size, and the PVDF membrane soaked in 100% methanol for approximately 2 minutes to activate.

Following electrophoresis, the gel tank apparatus was disassembled, the glass plate removed from the gel, and the stacking gel sliced off. The semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) was assembled by placing a plastic template containing a cut out region of equivalent size to the gel over the metal mesh (anode) of the transfer apparatus, and a small amount of transfer buffer was poured onto the mesh until the entire region was moistened. Two Whatman filter papers, pre-soaked in transfer buffer, were placed within the template, and the PVDF membrane laid on top. A pipette was then rolled gently over the PVDF membrane to remove any air bubbles, and the resolving gel placed on top of the PVDF membrane. A further two Whatman filter papers that had been pre-soaked in transfer buffer were placed on top of the resolving gel, and a pipette rolled over the Whatman filter paper to again expel any air

bubbles. The lid (cathode) of the semi-dry transfer apparatus was then placed on top of the sandwich and the protein transfer carried out at 0.13A for 90 minutes.

2.5.11 Protein Detection

Following Western blotting, the PVDF membrane was placed protein-side up and rinsed briefly in TBS-T before being blocked* overnight at 4°C in membrane blocking solution on a rocking platform. The blocking solution was then discarded, and the membrane incubated in the primary antibody (diluted in blocking solution or TBS-T) for 2 hours at room temperature, on a rocking platform.

The membrane was washed 6 times for 5 minutes in TBS-T at room temperature, and then incubated in an alkaline-phosphatase conjugated anti-mouse secondary antibody (diluted 1/2000 in blocking solution or TBS-T) for 1 hour at room temperature, on a rocking platform. The membrane was washed 6 times for 5 minutes in TBS-T at room temperature, and then rinsed briefly with TBS.

The protein was detected by incubating the membrane protein-side down on a glass plate with 500µL neat attophos substrate (ECF substrate, Amersham) for 2 minutes. The membrane was then scanned using a Typhoon 9410 (Amersham), using 488nm excitation.

* The blocking step was omitted if anti-phosphotyrosine antibody staining was being performed.

2.5.11.1 Western Blotting for Anti-Phosphotyrosine

Protein detection was performed as described in 2.5.11, with a combination of two primary antibodies used to detect α-phosphotyrosine: a mixture of 1/1000 PY20 (Santa Cruz Biotechnology Inc) and 1/2000 4G10 (Cell Signalling Technology®) in TBS-T.

2.5.11.2 Western Blotting for Anti-c-fms Protein

Protein detection was performed as described in 2.5.11, with the primary antibody (anti-human M-CSF R Antibody, R&D Systems) used at 2µg/mL in blocking buffer.

2.6 Statistical & Pharmacokinetic Data Analyses

Results are expressed as the mean $\pm$ the standard error of the mean (SEM). To determine whether differences between data points were statistically significant, the data was analysed using ANOVA. Differences were considered to be statistically significant when the probability value was <0.05.

The calculation of IC_{50} values was performed using GraphPad Prism® version 4.01 software (GraphPad, USA). The data was analysed using the Hill equation:

$$Y=100/(1+10^{((logIC50-X)*HillSlope)})$$

where Y is the level of inhibition, and X is logarithmic drug concentration.

The equation was fitted to unweighted data, with the use of non-linear regression analysis. IC_{50} values were not calculated when R^2 values were non-significant ($P<0.05$).

All experiments were performed a minimum of two times.

Chapter 3:

DIFFERENTIATION OF NORMAL & CML CD34^{+} CELLS

3.1 Introduction

Understanding the differentiation decisions of HSC along the various cellular pathways remains a major challenge, partly due to the mobile nature of the haemopoietic system. Although little is known about the regulation of the differentiation of HSC into mature blood cells, 2 models have been proposed. In the stochastic model it has been suggested that cells randomly differentiate towards various lineages, while in the deterministic model cells undergo a predictable program of differentiation (Pierelli *et al.* 2001).

Chronic myeloid leukaemia (CML) is a haemopoietic malignancy that originates in the stem cell pool and is characterised by elevated levels of mature granulocytes during chronic phase. As the disease progresses to blast crisis, immature cells are released from the bone marrow and circulate throughout the body. The disease is caused by a chromosomal abnormality, where a reciprocal translocation between abl on chromosome 9 and bcr on chromosome 22 to produce the Philadelphia chromosome results in the production of a fusion protein (bcr-abl) that displays constitutive tyrosine kinase activity (Rowley 1973; Groffen *et al.* 1984; Konopka *et al.* 1985; Ben-Neriah *et al.* 1986; Shtivelman *et al.* 1986). As a consequence, bcr-abl phosphorylates several cellular substrates, and multiple signal transduction pathways that have significant effects on cell growth and differentiation are activated (reviewed in Holyoake 2001a). Three main mechanisms have been proposed to be responsible for the transformation of cells in CML: a reduction in apoptosis (Bedi *et al.* 1994); altered adhesion to stromal cells (Gordon *et al.* 1987); and constitutively active mitogenic signalling (Puil *et al.* 1994; also reviewed in Deininger *et al.* 2000).

The effect of bcr-abl on the growth characteristics of CML progenitor cells is controversial. Although several groups have demonstrated that the proliferation of CML progenitors is similar to that of normal progenitor cells (Bedi *et al.* 1994; Thiele *et al.* 1997), other studies have suggested bcr-abl actively stimulates proliferation (Lepine and Messner 1983; Cortez *et al.* 1997; Jonuleit *et al.* 1998). The features of chronic phase CML are not consistent with increased proliferation and the discordant maturation model proposed by Clarkson and Strife (1988) suggests that the increased cellularity in CML results from extra rounds of cell division prior to terminal differentiation. Alternatively,

selective expansion of the leukaemic clone may result from decreased apoptosis of bcr-abl expressing cells (Bedi *et al.* 1994).

The existence of quiescent leukaemic HSC suggests that bcr-abl does not promote the entry of these cells into the cell cycle and that the cells retain, at least in part, normal responses to proliferative and differentiative signals (Holyoake, 1999 #77, reviewed in Kabarowski and Witte 2000). Although chronic phase CML cells are generally not hyperresponsive to cytokines, subtle differences in their response to combinations of growth factors, including stem cell factor, erythropoietin and GM-CSF, suggest that bcr-abl expression may activate signalling pathways involved in differentiation responses (reviewed in Kabarowski and Witte 2000).

The decision of a HSC to self renew or undergo differentiation appears to be extremely complex and is therefore likely to be regulated by a combination of intrinsic mechanisms specific to each cell, as well as extrinsic factors such as cytokines (Morrison *et al.* 1997). Whether cell division also plays a role in regulating HSC differentiation is unknown. Carboxy fluorescein diacetate, succinimidyl ester (CFSE) staining is a powerful tool by which the relationship between cell differentiation and division can be examined. As cells divide, CFSE is distributed equally between daughter cells (Lyons and Parish 1994) such that the fluorescence of daughter cells is half that of the parent cell. Using flow cytometry, the number of divisions the cells have undergone can therefore be tracked and correlated with the expression of other proteins. This approach was initially demonstrated in B cells, where the differentiation of these cells followed a defined program that was linked to cell division number and not culture duration (Hodgkin *et al.* 1996), and T cells, where cell division number following T cell activation was observed to be crucial in determining the type of cytokine produced (Gett and Hodgkin 1998).

The use of CFSE to track cell divisions has also been extended to normal $CD34^+$ cells, to demonstrate that the division kinetics of $CD34^+$ cells stimulated *in vitro* and *in vivo* are similar (Oostendorp *et al.* 2000). The phenotypic profile of $CD34^+$ cells expressing high and low levels of CFSE has also been carefully analysed, with CFSE-bright cells representing non/low proliferating cells and CFSE-dim cells representing cells that have

undergone several rounds of cell division (Pierelli *et al.* 1998; Pierelli *et al.* 2001). The CFSE-bright cells demonstrated a similar antigenic profile as freshly isolated CD34$^+$ cells that was indicative of their quiescent state, while CFSE-dim cells expressed lower levels of CD34 and gained expression of differentiation markers (Pierelli *et al.* 1998; Pierelli *et al.* 2001). From this data it was concluded that cells that retained high levels of CFSE staining maintained a primitive progenitor cell phenotype, while cells that had low levels of CFSE staining had undergone differentiation (Pierelli *et al.* 1998; Pierelli *et al.* 2001).

In order to gain a greater understanding of CML pathogenesis, it is fundamental to define alterations to cell behaviour at a biological level, in addition to defining the molecular mechanisms involved. A major hurdle in identifying abnormalities in the differentiation and proliferation of CML cells is our limited understanding of the factors that control the behaviour of HSC under normal conditions. In this chapter, the proliferation and differentiation of CD34$^+$ progenitor cells from normal donors is examined and compared to those from chronic phase CML patients. These studies were performed to examine the hypothesis that CML CD34$^+$ cells have an altered relationship between cell division and the acquisition of phenotypic markers.

A range of antibodies detecting cell surface molecules indicative of differentiation across the myeloid lineage were selected, and are shown in Table 3.1. CD34 was used to identify cells in the early stages of differentiation, CD33 and CD13 were used to identify myeloid progenitor cells, and CD11c, CD14 and CD15 were used to identify cells that were more mature. This analysis was performed for 3 normal donors and 3 CML patients and the data used to determine whether changes in the expression of markers with cell division were consistent for all normal donors, or were donor specific, and whether the behaviour of CD34$^+$ cells from CML patients was altered relative to normal donors. A greater understanding of the mechanisms behind CML will essentially aid in the development of therapeutics targeted to this haematological disease.

Table 3.1. Markers of differentiation, their function, & the cell types on which they are expressed.

Marker	Molecule/Function	Cellular Expression
CD11c	Adhesion glycoprotein.	Natural killer cells, subset of T and B cells, monocytes, granulocytes and macrophages.
CD13	Aminopeptidase N. Involved in the metabolism of many regulatory peptides.	Granulocytic and monocytic cells, mast cells, GM-progenitor cells.
CD14	Glycosylphosphatidylinositol (GPI). High affinity cell-surface receptor for complexes of LPS.	Monocytes (high levels), interfollicular macrophages, reticular dendritic cells, Langerhans cells.
CD15	3-fucosyl-N-acetyllactosamine (X-hapten). Plays a role in mediating phagocytosis and chemotaxis.	Granulocytes (including neutrophils and eosinophils), monocytes.
CD33	67kD type I transmembrane glycoprotein. Functions as an adhesion molecule.	Monocytes, activated T cells, myeloid progenitors, mast cells.
CD34	O-glycosylated transmembrane glycoprotein. May play a role in signal transduction (part of CD34 antigen is target for phosphorylation by protein kinase C) and adhesion.	Haematopoietic progenitor cells, vascular endothelium, some tissue fibroblasts.
CD36	88kD glycoprotein IV receptor for extracellular matrix proteins such as collagen.	Platelets, endothelial cells, monocytes, early erythroid cells.
CD38	45 kD type II single-chain transmembrane glycoprotein.	Thymocytes, activated T cells, terminally differentiated B cells (plasma cells), monocytes, macrophages, dendritic cells.
HLA-DR	Human MHC II transmembrane glycoprotein. Important in cellular interaction during antigen presentation.	Antigen presenting cells, B cells, monocytes, macrophages, thymic epithelial cells, activated T cells.

Information obtained from:

Schlossman *et al.* (ed). Leucocyte typing V: white cell differentiation antigens: proceedings of the fifth international workshop and conference held in Boston, USA, 3-7 November 1993.

3.2 Results

CD34$^+$ cells from normal donors ("Normals") and chronic phase CML patients ("CMLs") were isolated from the bone marrow or peripheral blood respectively, stained with CFSE, and cultured over a 4 day period. These cultures were established in serum deprived media that was supplemented with a combination of haemopoietic growth factors (IL-3, IL-6, G-CSF, GM-CSF, thrombopoietin and Flt-3) that are known to be potent stimulators of proliferation and differentiation along the myeloid lineage (Haylock *et al.* 1992; Li *et al.* 2000). CML patients whose CD34$^+$ cells were at least 90% positive for the Philadelphia (Ph) chromosome were selected for use in this study (CML A = 96% Ph$^+$; CML B = 93% Ph$^+$; CML C = 93% Ph$^+$).

3.2.1 Comparing the Proliferation of Normal & CML CD34$^+$ Cells

CFSE labelled cells from normal donors and CML patients were removed from culture at 24 hour time points and analysed for fluorescence using flow cytometry. Cytomics^{TM} RXP Analysis Software was used to quantify the percentage of cells within each given division cycle to enable calculation of various parameters indicative of proliferative characteristics. This included calculation of the percentage of starting cells that entered division, the proliferation index and the distribution of cells across each cell division (Figures 3.1 and 3.2). Data from 3 CML patients was analysed, in addition to averaged data from 3 normal donors ("normal controls").

3.2.1.1 Comparison of the Percentage of Starting Cells Entering Division in Normal Donors & CML Patients

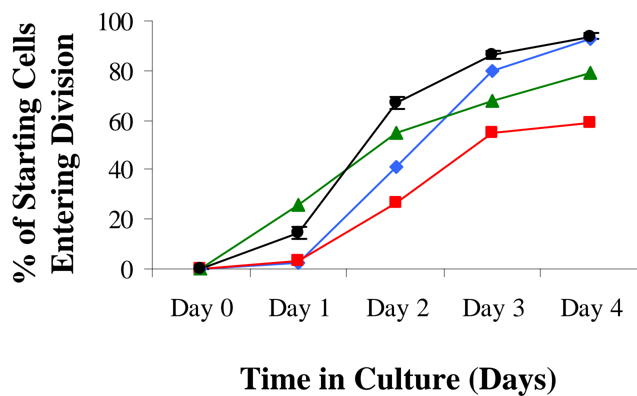
The percentage of starting cells that entered cell division was calculated according to the equation described in section 2.3.6.2. A similar profile for the percentage of starting cells that had entered division was observed for all normal donors, while variation was observed between CML patients, as well as between CML patients and the normal control (Figure 3.1A).

On day 1 of culture, 15% of starting cells from the normal controls had entered division and this increased to 95% by day 4 of culture (Figure 3.1A). In CML A, 75% fewer starting cells had entered division on day 1 of culture, but by day 3 of culture the number

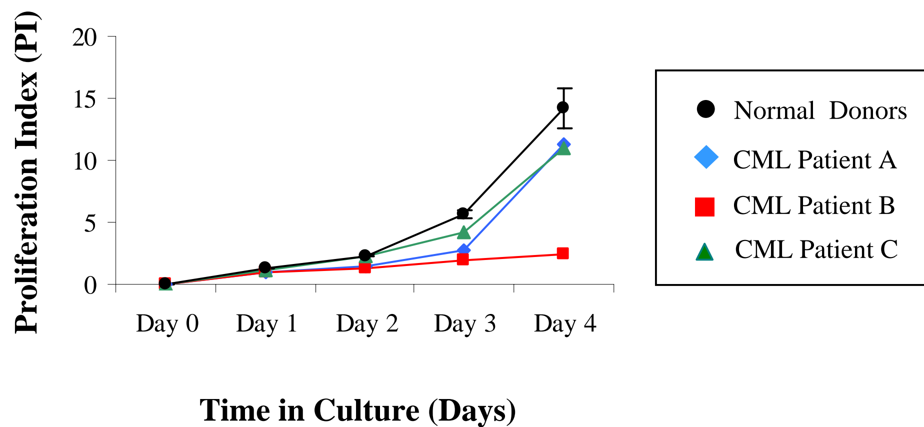
Figure 3.1 Analysis of the Growth Characteristics of Normal and CML $CD34^+$ Cells in Cultures Stimulated with IL-3, IL-6, G-CSF, GM-CSF, TPO and Flt-3.

$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CFSE fluorescence using flow cytometry. The percentage of starting cells that entered division (**A**), the proliferation index (**B**) and viability (**C**) of cells was calculated and plotted over 4 days of culture as described in the methods. Individual results from 3 CML patients are displayed, along with averaged results from 3 normal donors. A decrease in the percentage of starting cells that entered division was observed in all CML patients on day 2 of culture compared to the normal control, and this was maintained over days 3 and 4 of culture for CML patients B and C (**A**). No difference in the percentage of starting cells that entered division was observed on day 4 of culture for CML patient A (**A**). A decrease in the proliferation index of CML cells was apparent on days 3 and 4 of culture for all patients, but was most pronounced in CML patient B (**B**). The viability of cultured CML cells was also lower than normal controls on each of the 4 days of culture (**C**).

A



B



C

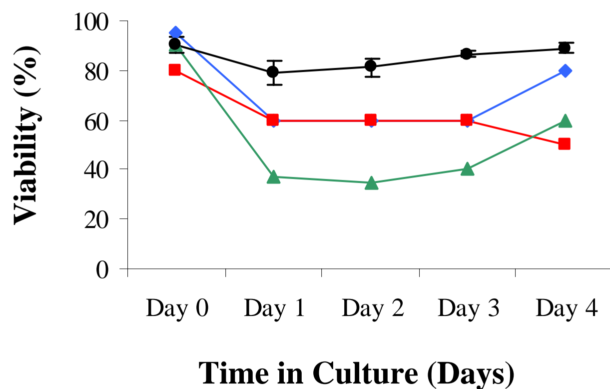
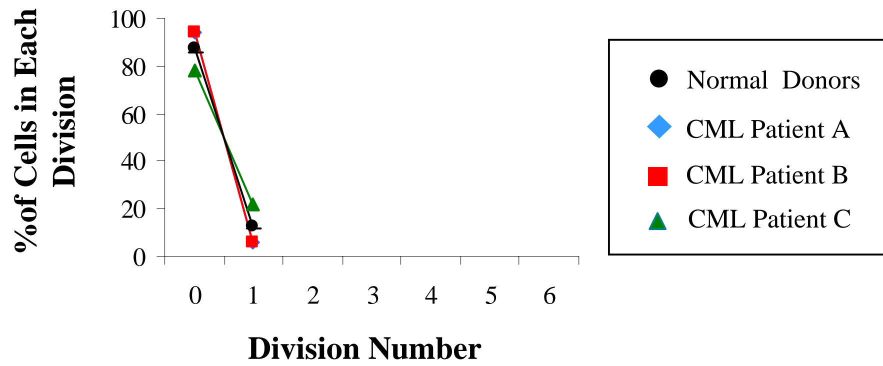


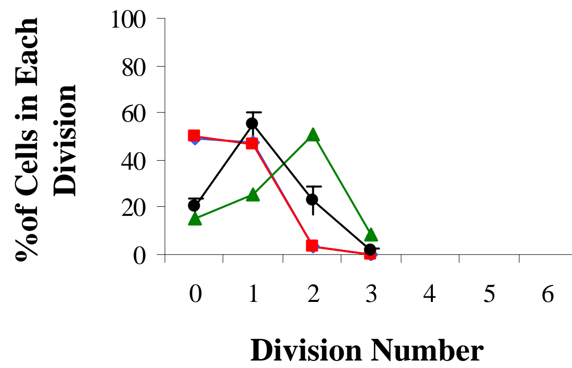
Figure 3.2 Proliferation of Normal and CML CD34$^+$ Cells in Cultures Stimulated with IL-3, IL-6, G-CSF, GM-CSF, TPO and Flt-3.

CD34$^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CFSE fluorescence using flow cytometry. The proportion of cells within each division was calculated and plotted over 4 days of culture. Individual results from 3 CML patients are displayed, along with averaged results from 3 normal donors. No difference in the distribution of cells within each division was observed on day 1 of culture for CML patients and the normal control. On day 2 of culture, an increase in the percentage of cells in division 0 was apparent in CML patients A and B, in addition to a decrease in the percentage of cells in division 2. In contrast, an increase in the percentage of cells in division 2 was observed on day 2 of culture in CML patient C. By day 4 of culture, the profile for the distribution of cells across each division in CML patients A and C was similar to the normal control. In CML patient B, an increase in the percentage of cells in divisions 0-2 was observed on day 4 of culture.

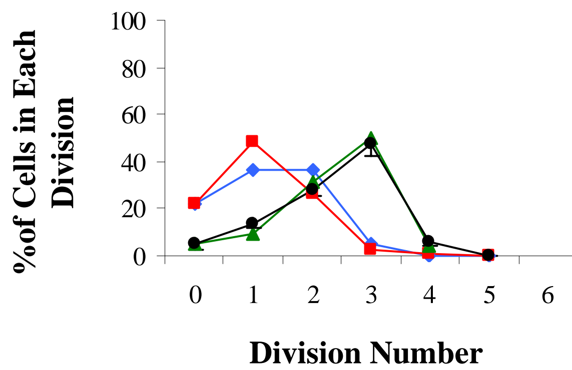
Day 1



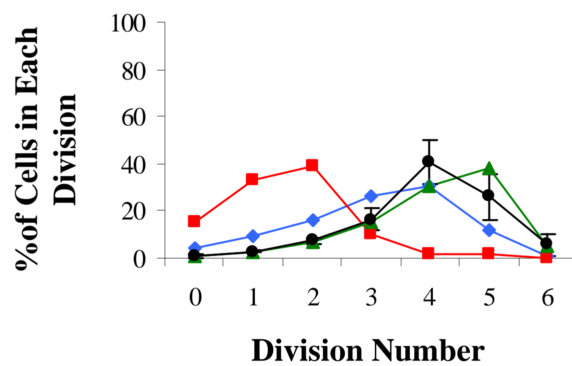
Day 2



Day 3



Day 4



of starting cells that had entered division was similar to the normal controls (Figure 3.1A). Out of the 3 patients examined, CML B exhibited the greatest reduction in the percentage of starting cells that entered division. For example, 75% less starting cells entered division on day 1 of culture compared to the normal controls and this decreased entry of cells into the cell cycle was maintained over the 4 days of culture. In CML C, 50% more cells had entered division on day 1 of culture compared to the normal controls and by day 4 of culture 20% fewer cells had entered the cell cycle (Figure 3.1A).

3.2.1.2 Comparison of the Proliferation Index of Normal & CML CD34$^+$ Cells

The proliferation index (PI) over 4 days of culture was calculated according to the equation described in section 2.3.6.2 to further examine the proliferative behaviour of normal and CML CD34$^+$ cells (Figure 3.1B). The PI of cells from normal donors was found to be similar, as demonstrated by the small standard error values. Little difference in the PI was also observed between CML patients, and between CML patients and normal controls, on days 1 and 2 of culture. The PI of cells from CMLs A and C were similar over the 4 days of culture and was approximately 30% lower than the PI value for normal controls on days 3 and 4 of culture. A markedly different PI profile was observed in CML B with the PI value on day 4 of culture 80-85% lower than either the normal controls or CMLs A and C (Figure 3.1B).

3.2.1.3 Comparison of the Viability of Cultured Cells from Normal Donors & CML Patients

The viability of cells was examined over 4 days of culture to determine whether variations in proliferation kinetics may be linked to an increase in cell death (Figure 3.1C). The viability of cells in the normal controls was consistently 80-90% over the culture period. Similar viabilities were observed in freshly isolated cells from CML patients as the normal controls, but by day 1 of culture a 25-60% decrease in the viability of CML cells was observed and was maintained over days 2 and 3 of culture. This decrease in viability was most marked in CML C where a 60% decrease in viability was observed. By day 4 of culture an increase in the viability of cultured cells in CMLs A and C was seen, with the viability estimated to be 80% in CML A and 60% in CML C (Figure 3.1C).

3.2.1.4 Comparison of the Distribution of Cells Across Cell Divisions in Normal Donors & CML Patients

The proliferative behaviour of normal and CML CD34$^+$ cells was also compared by examining the distribution of cells across each division (Figure 3.2). This was performed to determine whether CML cells that commenced cell division continued to proliferate at a similar rate to their normal counterparts. The percentage of cells within each division was calculated in 3 CML patients over 4 days of culture and compared to averaged data from 3 normal donors (Figure 3.2).

On day 1 of culture, no difference in the percentage of cells in divisions 0 and 1 were observed between CML patients and the normal controls, or between CML patients themselves (Figure 3.2). On days 2 and 3 of culture similar profiles were observed for CMLs A and B, although these profiles were different to the normal control with more cells remaining undivided and fewer cells present in later divisions such as division 3 on day 3 of culture. In contrast, cells from CML C appeared to be cycling at a faster rate compared to the normal controls or CMLs A and B on day 2 of culture, with fewer cells in division 1 and a greater percentage of cells in division 2 compared to the normal controls (Figure 3.2).

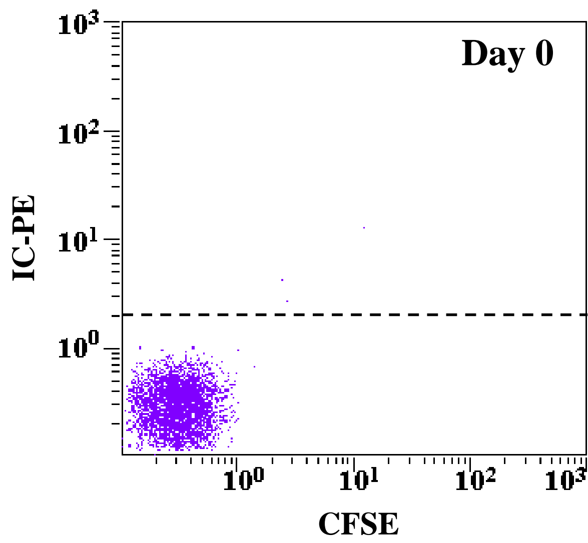
The profiles for the percentage of cells across each division on day 4 of culture were similar for CMLs A and C and the normal donors. In CML B, a greater percentage of cells remained in early division cycles (Figure 3.2).

3.2.2 Comparison of the Differentiation Program of Normal and CML CD34$^+$ Cells using Dye Dilution

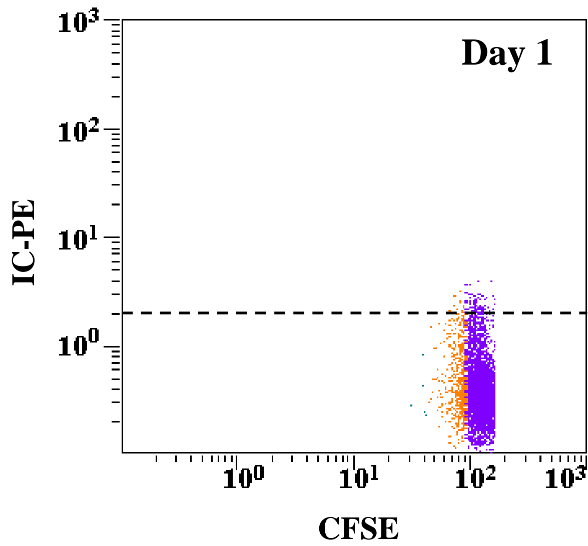
In addition to using CFSE staining to compare the proliferation kinetics of normal and CML CD34$^+$ cells, the phenotype of cells in relation to cell division was examined. CFSE labelled cells were removed from culture at 24 hour time points and stained with antibodies specific for cell surface molecules indicative of cellular differentiation as listed in Table 3.1. The background fluorescence level of negative cells was determined using isotype controls, and dashed lines on dot plots delineated the cut off point between positive and negative cells (Figure 3.3). It should be noted that although complex expression profiles

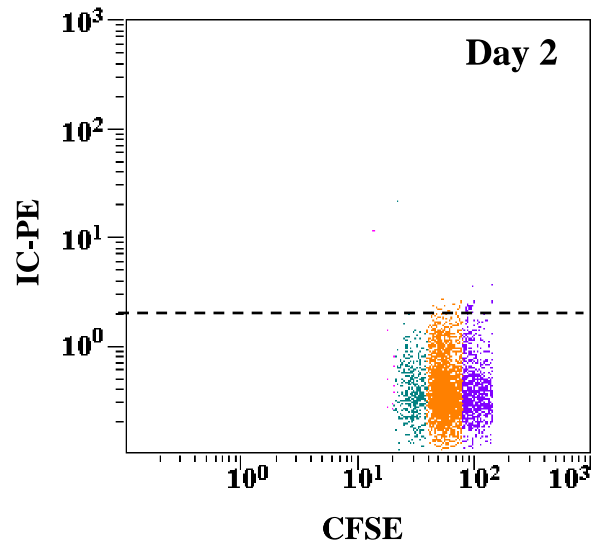
Figure 3.3 Isotype Control Staining of CD34$^+$ Cells from Normal Donor A.

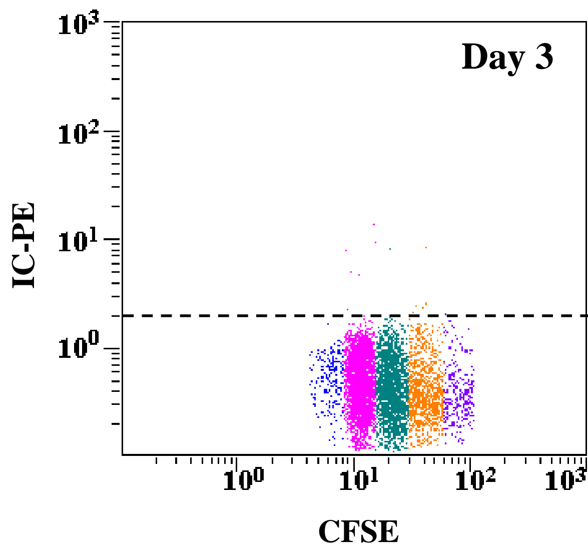
CD34$^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for non-specific staining of an isotype control using flow cytometry. Dot-plot analysis of fluorescence profiles was used to examine the effect of cell division on non-specific staining, with the dashed line delineating the cut off point between positive and negative staining for PE-conjugated antibodies. Coloured dots were used to distinguish cell division number. Results represent an example of flow cytometric data from normal donor A.

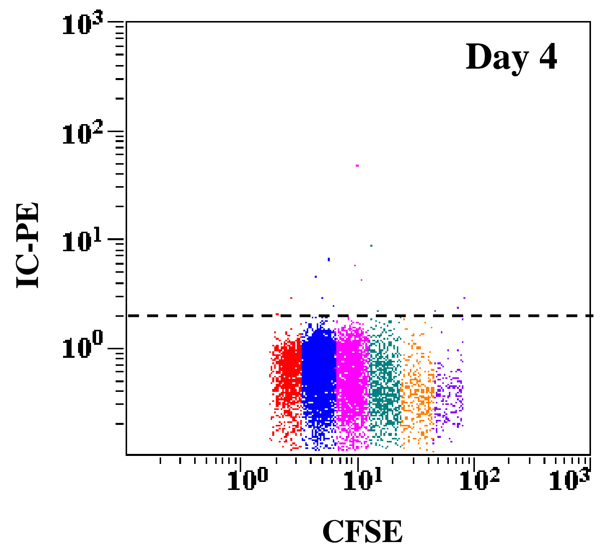

Day 0
IC-PE
CFSE

Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6


Day 1
IC-PE
CFSE


Day 2
IC-PE
CFSE


Day 3
IC-PE
CFSE


Day 4
IC-PE
CFSE

were observed fro some phenotypic markers and suggested the presence of different cell populations, this unbiased approach to expression analysis was applied to all samples for consistency.

Simultaneous analysis of CFSE fluorescence and the expression of phenotypic markers enabled concurrent analysis of changes in cell phenotype with cell division. Cytomics™ RXP Analysis Software was used to quantify the percentage of cells within each given division cycle that were positive for the phenotypic marker of interest, and this data was modelled graphically to allow comparison between marker expression and cell division number over each of the 4 days in culture. This analysis was performed to determine whether changes in marker expression were related to cell division number and/or length of time in culture.

3.2.2.1 Comparison of the Phenotype of Freshly Isolated Normal & CML CD34+ Cells

CD34+ cells from normal donors and CML patients were analysed for the expression of phenotypic markers at the initiation of the culture by calculating the percentage of cells that expressed the marker of interest, as well as the mean fluorescence intensity (MFI) of the positive population. This was performed to determine whether the phenotype of freshly isolated CD34+ cells was similar in normal donors and/or CML patients. Data from 3 normal donors was averaged to simplify the comparison and the results are presented in Table 3.2.

In normal donors, the percentage of cells expressing each of the markers of interest, as well as the level of expression of these markers, was similar for all donors (Table 3.2). This was reflected by the low standard error values, and excludes the percentage of cells expressing CD13 which varied markedly between the normal donors.

The percentage of CML CD34+ cells that expressed CD38, CD14, CD11c and CD15 was similar between all CML patients and between the CML patients and normal controls (Table 3.2). Variation in the percentage of cells expressing CD33 relative to the normal control was observed in all CML patients, while other differences in phenotype were

Table 3.2. Phenotype of Freshly Isolated CD34^{+} Cells from Normal Donors and CML Patients.

Marker	Normals		CML A		CML B		CML C	
	%	MFI	%	MFI	%	MFI	%	MFI
CD34	94±2.7	8.1±0.5	99	9.7	98	22.1	98	21.7
CD38	98±0.6	18.3±2.1	91	8.7	98	13.6	85	9.0
HLA-DR	94±2.5	18.1±7.0	98	16.8	11	14.8	95	21.4
CD33	47±3.5	6.3±1.4	33	2.5	98	14.1	66	4.4
CD13	67±20	8.6±2.6	46	3.5	78	3.2	78	6.6
CD36	5±1.5	10.3±6.3	8	5.0	5	4.3	34	4.4
CD14	0	-	0	-	0	-	2	4.9
CD11c	11±3.1	8.5±4.6	4	2.3	3	2.5	6	2.9
CD15	0	-	0	-	0	-	0	-

% = Percentage of cells expressing the marker of interest.

MFI = Mean Fluorescence Intensity.

■ = expression significantly different to normal controls.

specific to particular CML patients. For example, a 50% decrease in the level of CD38 expression was observed in CML A and 90% fewer cells expressed HLA-DR in CML B. In CML C, a 610% increase in the percentage of cells that expressed CD36 and a 50% decrease in the level of CD38 expression was observed compared to normal controls (Table 3.2).

3.2.2.2 CD34 Expression

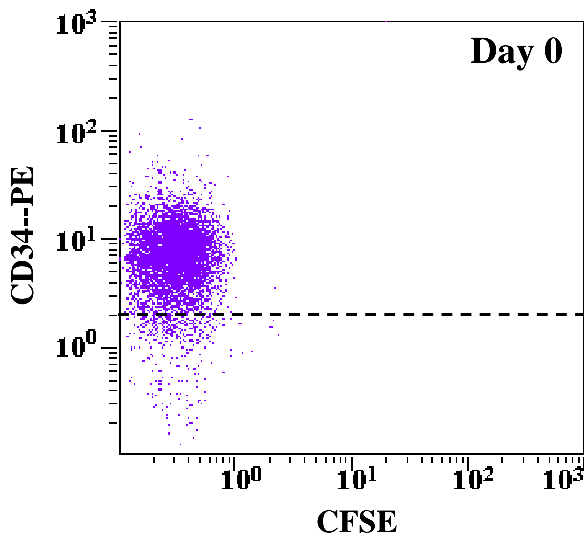
Changes in the level of CD34 expression with cell division events for normal donor A are shown in Figure 3.4A. At the initiation of the culture, the mean fluorescence intensity (MFI) of the positive population was approximately 8 fluorescence units. Following 1 day of culture undivided cells expressed the same level of CD34 as freshly isolated cells and the MFI of cells that had undergone 1 division increased to 10 fluorescence units. On day 2 of culture, cells that had undergone 3 divisions were present and the maximum level of CD34 expression increased from 30 units on days 0 and 1 of culture to 100 units (Figure 3.4A). This increase in the maximum level of CD34 expression was maintained over days 3 and 4 of culture until division 4, where the MFI of cells within each cell division decreased with an increase in cell division. An increase in the proportion of CD34 negative cells was also observed with an increase in culture duration and cell division (Figure 3.4A).

Analysis of changes in the percentage of $CD34^+$ cells with cell division for 3 normal donors and 3 CML patients is shown in Figure 3.4B. A similar profile for the percentage of cells expressing CD34 over 4 days of culture was observed in Nomals A and D, with a decrease in the percentage of $CD34^+$ cells occurring with an increase in culture duration as well as an increase in cell division after division 3 (Figure 3.4B). A different profile was observed for Normal C, as the percentage of undivided cells expressing $CD34^+$ cells decreased with culture duration on days 1-3 but increased on day 4 (Figure 3.4B).

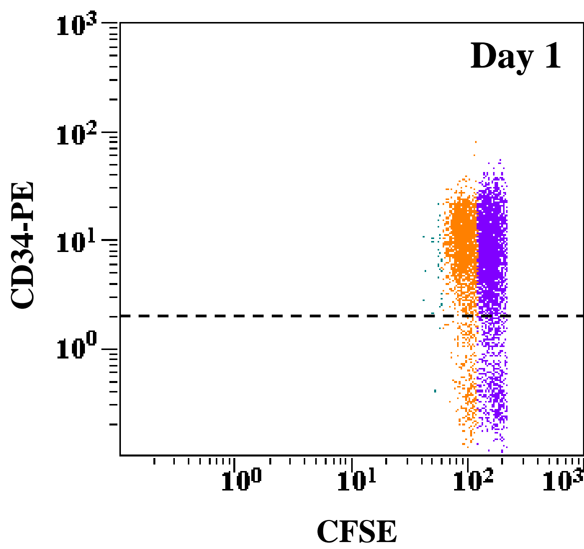
Similar profiles for the percentage of cells expressing CD34 were observed in CMLs A and B over 4 days of culture, with the percentage of cells expressing CD34 decreasing with an increase in cell division number but remaining unchanged with culture duration (Figure 3.4B). A different trend was seen in CML C, as the percentage of $CD34^+$ cells increased

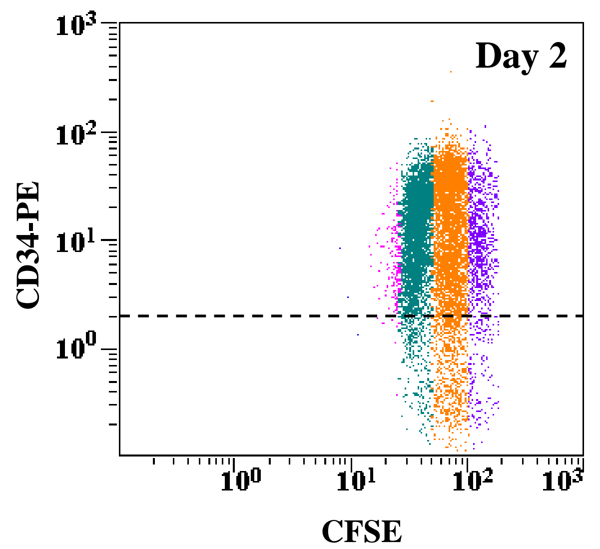
Figure 3.4A Change in CD34 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

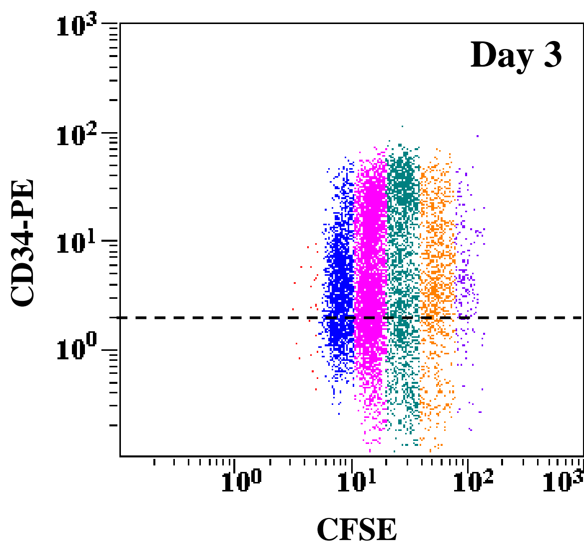
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD34 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD34, with the dashed line delineating cells that were positive and negative for CD34 expression, and the coloured dots distinguishing cell division number. Cells underwent 6 rounds of cell division over the 4 days of culture, and a decrease in the level of CD34 expression was observed with an increase in cell division number and an increase in culture duration. Results represent an example of flow cytometric data from normal donor A.



Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6







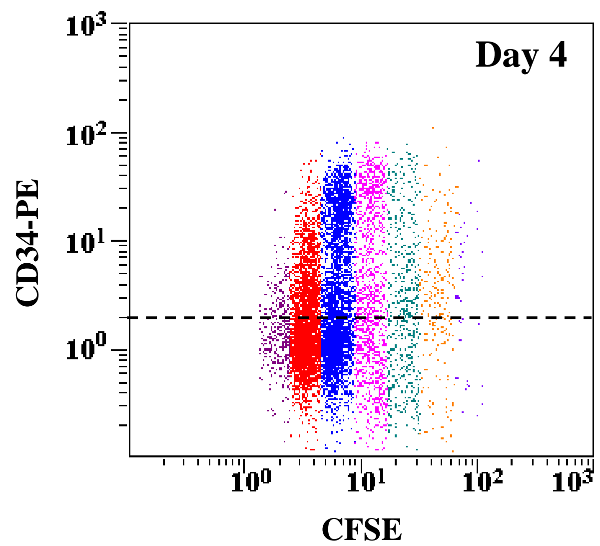
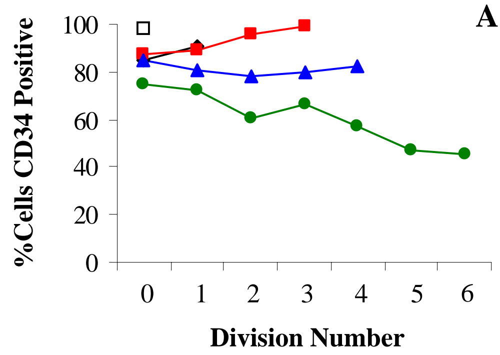


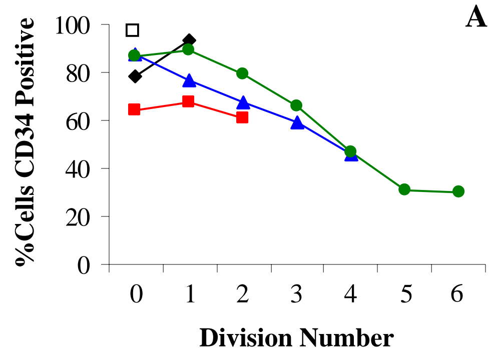
Figure 3.4B Changes in the Percentage of Cells Expressing CD34 with Cell Division & Culture Duration in Normal Donors and CML Patients.

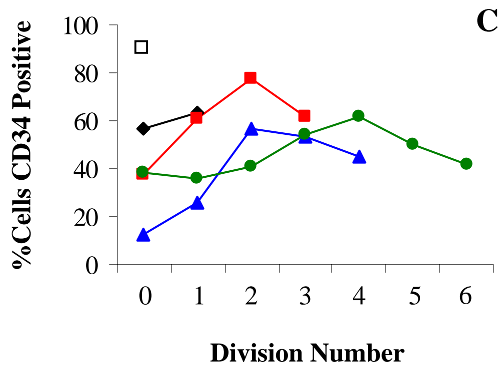
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD34 expression using flow cytometry. The percentage of cells that were positive for CD34 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. The percentage of cells that expressed CD34 in normal donors decreased with an increase in culture duration, but was largely unaffected by cell division number. In contrast, the percentage of $CD34^+$ cells decreased with both culture duration and cell division number in CML patients A and B. In CML patient C, an increase in the percentage of cells that expressed CD34 in division 0 was observed on day 4 of culture, and correspondingly decreased with an increase in cell division number.

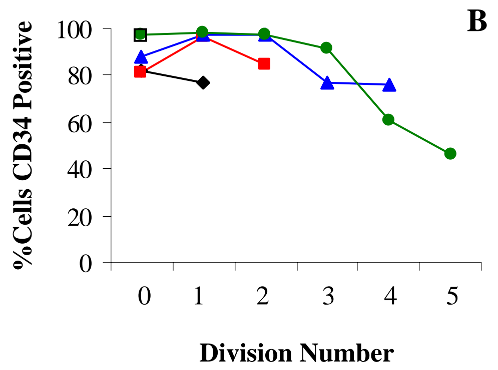
Normal Donors

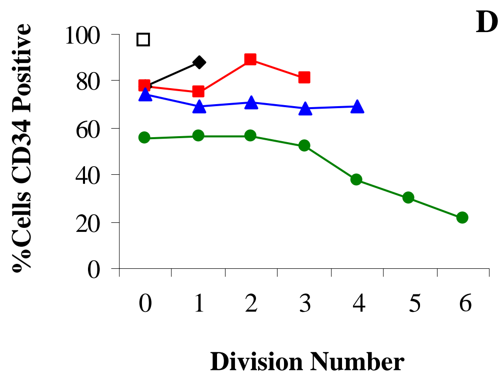
CML Patients

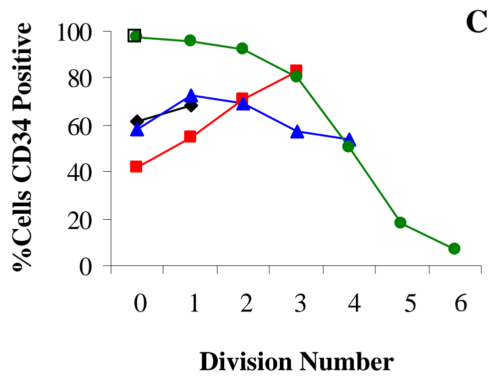












from 54±6% on days 1-3 of culture to 97% on day 4 of culture. A decrease in the percentage of cells expressing CD34 in CML C was also seen with an increase in cell division number on day 4 of culture (Figure 3.4B).

Comparing the profiles of CD34 expression in normal donors to CML patients identified that the percentage of undivided cells expressing CD34 on day 4 of culture was higher in CML patients (94±3%) than in normal donors (56±10%) on day 4 of culture (Figure 3.4B). In normal donors, this was associated with a decrease in the percentage of cells expressing CD34 with culture duration, whereas in CML patients the percentage of $CD34^+$ cells increased with culture duration (CML C) or remained unchanged (CMLs A and B). By divisions 5 and 6, the percentage of cells expressing CD34 was similar in CML patients (26±7%) and normal donors (39±5%).

3.2.2.3 CD38 Expression

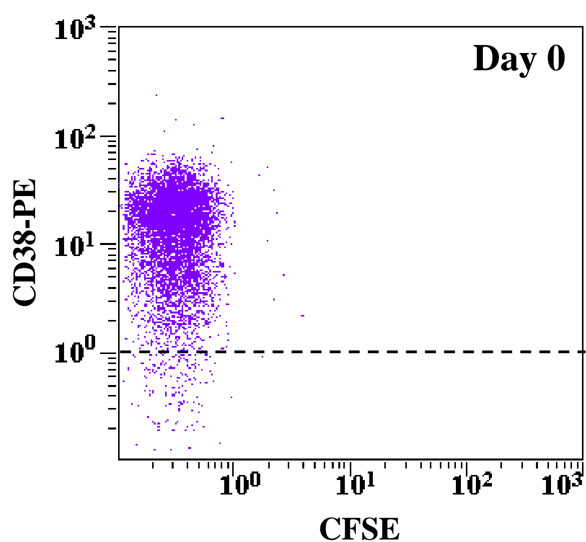
Figure 3.5A shows the profile of CD38 expression with cell division over 4 days of culture for normal donor A. In freshly isolated cells, 2 populations of cells were evident based on high (MFI=20) and medium (MFI=5) levels of expression. Following culture, CD38 expression that ranged from medium to high levels was observed, and a decrease in the MFI of this population was observed with an increase in cell division on days 1-4 of culture (Figure 3.5A). The decrease in the level of CD38 expression with cell division was observed until division 6, where few $CD38^+$ cells were observed (Figure 3.5A).

A similar profile for the percentage of cells expressing CD38 over 4 days of culture was seen in Normals A, B and C (Figure 3.5B). In particular the percentage of $CD38^+$ cells remained relatively unchanged over the culture, excluding divisions 5 and 6 in Normal A where 25% and 70% decreases in the percentage of $CD38^+$ cells were seen in divisions 4 and 5 respectively (Figure 3.5B). The percentage of undivided cells expressing CD38 was also consistent across all normal donors (90±2.4%) (Figure 3.5B).

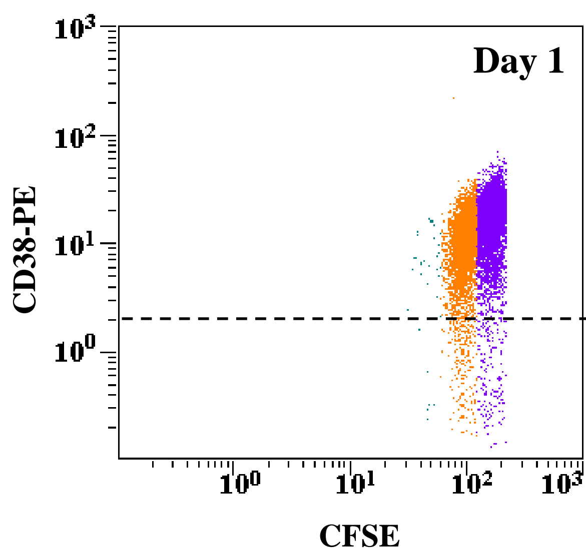
Although the percentage of freshly isolated cells that expressed CD38 was similar in all CML patients (91±4%), patient variability was observed with respect to the effect of culture duration and cell division on the percentage of $CD38^+$ cells (Figure 3.5B). For

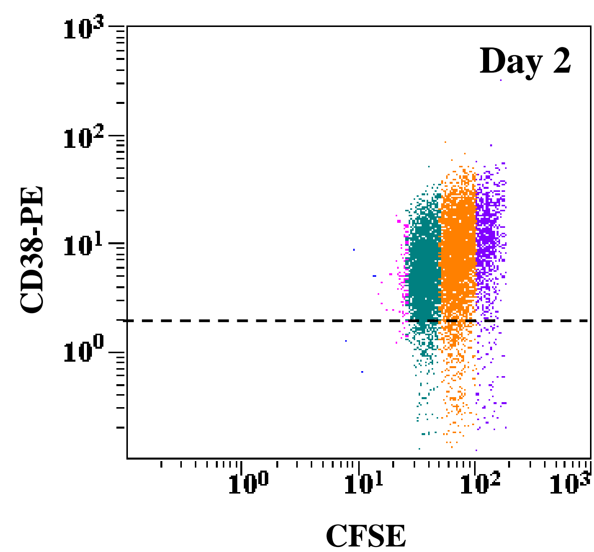
Figure 3.5A Change in CD38 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

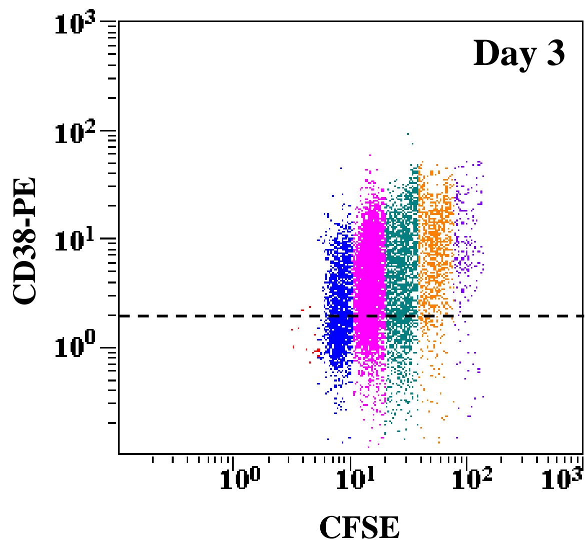
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD38 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD38, with the dashed line delineating cells that were positive and negative for CD38 expression, and the coloured dots distinguishing cell division number. Cells underwent 6 rounds of division over the 4 days of culture. The majority of freshly isolated cells expressed CD38, with 2 populations evident based on high (mean fluorescence intensity (MFI) = 20) and medium (MFI=5) levels of expression. Following culture, only 1 population of CD38$^+$ cells was observed with expression that ranged from medium to high levels. A decrease in the MFI of this population was observed with an increase in cell division on days 1-4 of culture, and occurred until division 6, where few CD38$^+$ cells were observed. Results represent an example of flow cytometric data from normal donor A.



Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6







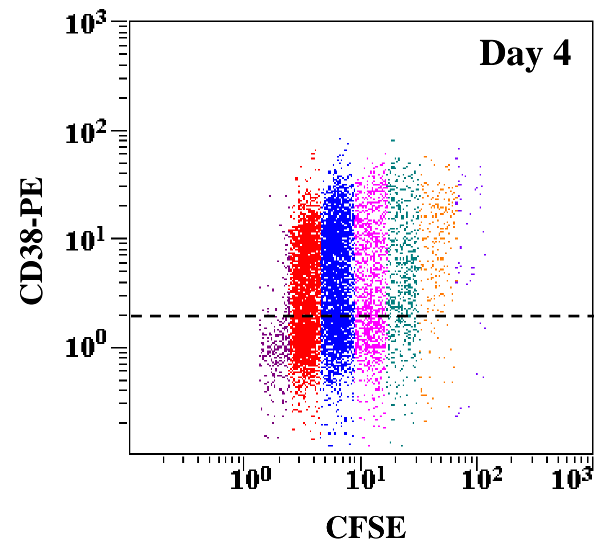
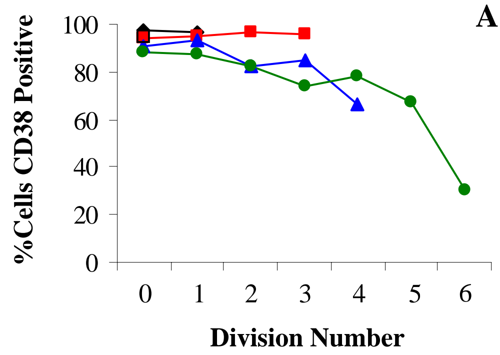
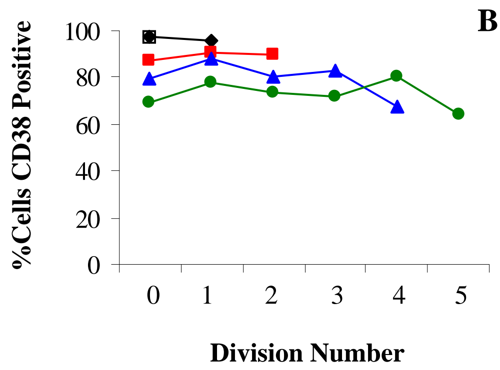


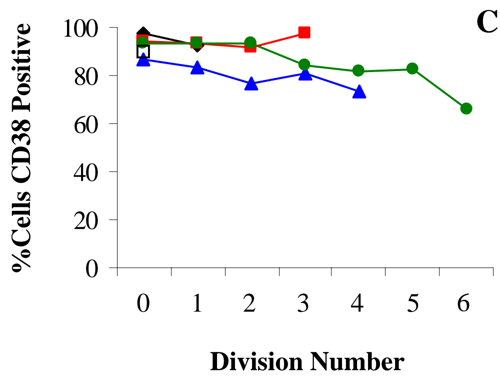
Figure 3.5B Changes in the Percentage of Cells Expressing CD38 with Cell Division & Culture Duration in Normal Donors and CML Patients.

$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD38 expression using flow cytometry. The percentage of cells that were positive for CD38 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. The percentage of $CD38^+$ cells was unaffected by culture duration, but decreased with cell division in divisions 5-6 in all normal donors. In CML patients A and C, the percentage of $CD38^+$ cells changed with cell division number and/or culture duration, while in CML patient B, the percentage of $CD38^+$ cells increased with culture duration but was relatively unaffected by cell division.

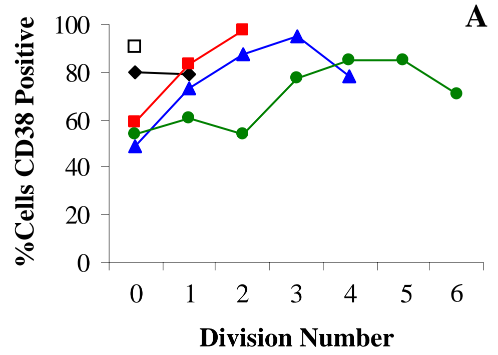
Normal Donors

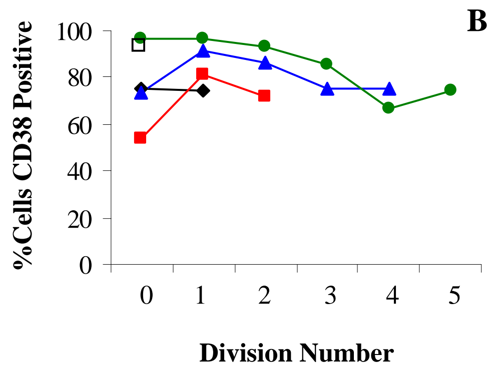


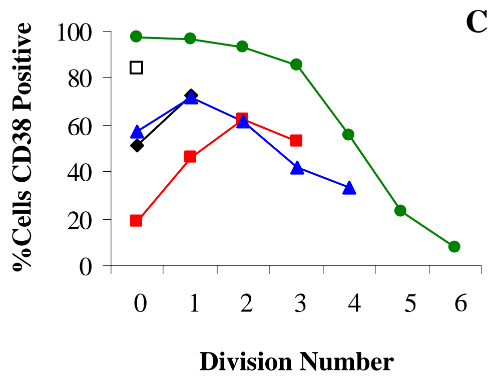




CML Patients







example, an increase in the percentage of $CD38^+$ cells was seen with cell division in CML A, in addition to a decrease in the percentage of $CD38^+$ cells with culture duration. Culture duration had a much greater influence on the percentage of cells expressing CD38 in CML C, with the percentage of cells expressing CD38 in division 0 decreasing between days 1 and 2 of culture and increasing across days 3 and 4 of culture. In CML C, cell division had a marked effect on the percentage of cells expressing CD38 as a 25-50% increase in the percentage of cells expressing CD38 occurred between divisions 0 and 1 on days 1-3 of culture in addition to a decrease occurring after division 2 and/or 3 on days 2-4 of culture. In comparison to CMLs A and C, the percentage of cells expressing CD38 in CML B was relatively unchanged by cell division or culture duration (Figure 3.5B).

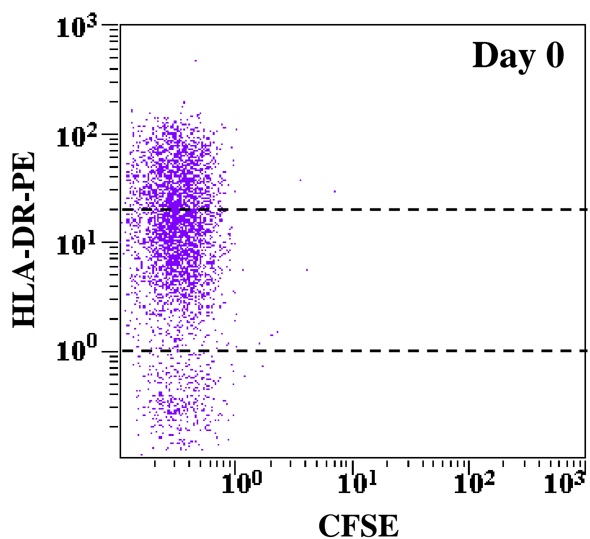
A comparison of the profile of CD38 expression between normal donors and CML patients revealed that the percentage of undivided cells expressing CD38 in CML patients on days 1-3 of culture was significantly lower than that observed in normal donors (Figure 3.5B). For example, an average of 92% of cells were $CD38^+$ in normal donors, versus 58% of cells from CML patients ($p<0.01$) (Figure 3.5B). Culture duration also had a greater impact on the percentage of cells expressing CD38 from CML patients than normal donors, although by day 4 of culture 97% of undivided cells were $CD38^+$ in CMLs B and C, similar to the level seen in Normals A and C (Figure 3.5B).

3.2.2.4 HLA-DR Expression

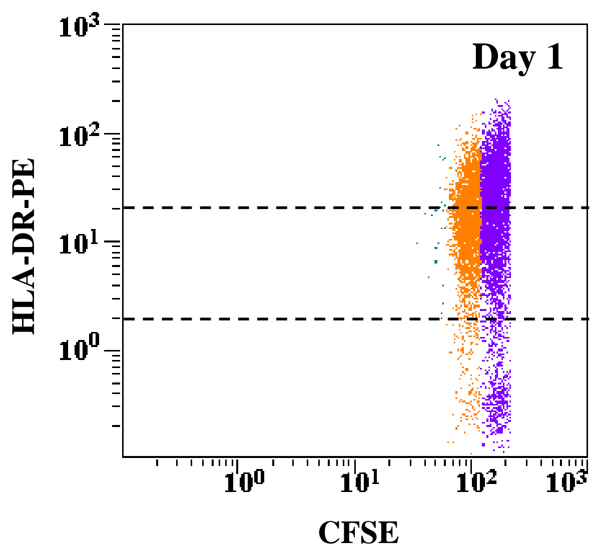
An example of the profile of HLA-DR expression by $CD34^+$ cells over 4 days of culture is displayed for normal donor A (Figure 3.6A). HLA-DR expression on freshly isolated cells ranged from 0-150 units and the MFI of the positive population was approximately 30 units. After 1 day of culture the cells had undergone 1 division cycle. This was accompanied by a decrease in the maximum level of HLA-DR expression from 150 units in undivided cells to 100 units in division 1, as well as a decrease in the MFI value from an MFI of 30 units in undivided cells to an MFI of 20 units in division 1. A decrease in the level of HLA-DR expression was observed with an increase in cell division over the 4 days of culture, such that by division 6 on day 4 of culture, the MFI was approximately 10 units. Variation in the MFI of undivided cells was observed over the 4 days of culture, with the MFI on day 0 measuring 30 units, and on day 3 measuring 95 units. Since changes in

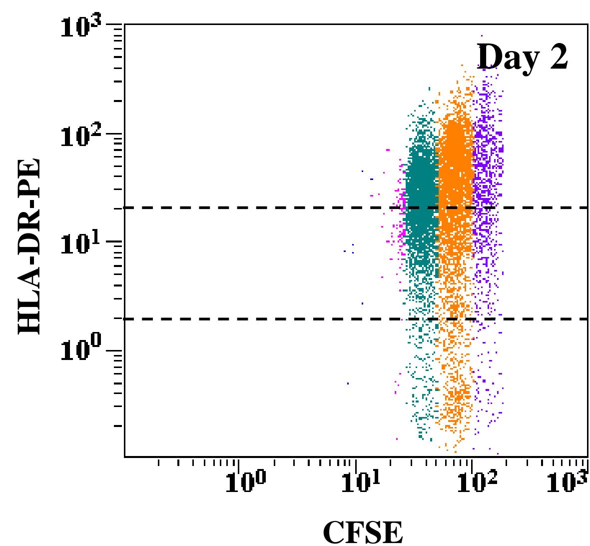
Figure 3.6A Change in HLA-DR Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

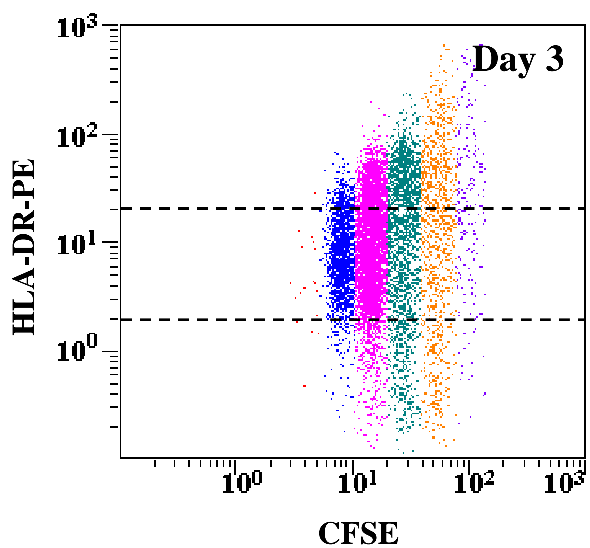
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for HLA-DR expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of HLA-DR, with the dashed line delineating cells that were positive (high or medium) and negative for HLA-DR expression, and the coloured dots distinguishing cell division number. Cells underwent 6 rounds of cell division over 4 days of culture. Cells expressing high and medium levels of HLA-DR, as well as a negative population, were observed on freshly isolated cells. After 1 cell division, a decrease in the mean fluorescence intensity (MFI) as well as the maximum fluorescence of the positive population was observed. A decrease in the MFI was observed with cell division over the 4 days of culture. Results represent an example of flow cytometric data from normal donor A.

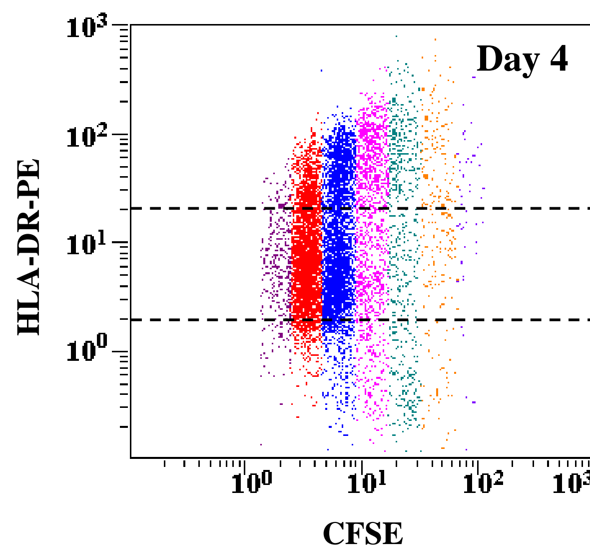


Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6









HLA-DR expression over culture were associated with a decrease in expression levels rather than a loss of expression, division analysis was applied to cells that expressed high and medium levels of HLA-DR, as depicted by the dashed lines (Figure 3.6A).

3.2.2.4.1 HLA-DR^{high} Expression

The expression profile for cells expressing HLA-DR^{high} was similar in all 3 normal donors. In particular, 62±1.9% of cells expressed HLA-DR^{high} in division 0 and this percentage decreased with cell division (Figure 3.6B). By division cycle 5 or 6, approximately 20% of cells from normal donors expressed high levels of HLA-DR.

Similar expression profiles were also observed across CML patients, with low levels of cells expressing HLA-DR^{high} over the culture period (Figure 3.6B). In CML A approximately 25% of cells in division 0 expressed HLA-DR^{high} on days 1 and 2 of culture and this percentage decreased with an increase in cell division and culture duration to an average of 4% after division 1 (Figure 3.6B).

The profile of HLA-DR^{high} expression was markedly different in CML patients compared to normal donors (Figure 3.6B). In particular, the percentage of undivided cells expressing HLA-DR^{high} was approximately 90% lower in CML patients than normal donors.

3.2.2.4.2 HLA-DR^{medium} Expression

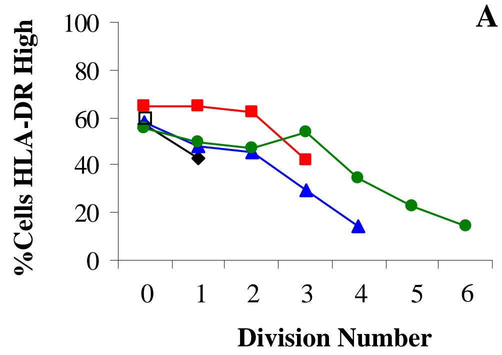
The profile of cells expressing HLA-DR^{medium} was comparable in all normal donors as the percentage of undivided cells expressing HLA-DR^{medium} was similar (29±1.7%), and the percentage of cells expressing HLA-DR^{medium} increased with cell division number but was unaffected by culture duration (Figure 3.6C). The percentage of cells expressing HLA-DR in division 6 was also similar in all normal donors, ranging from 76% in Normal A to 87% in Normal D.

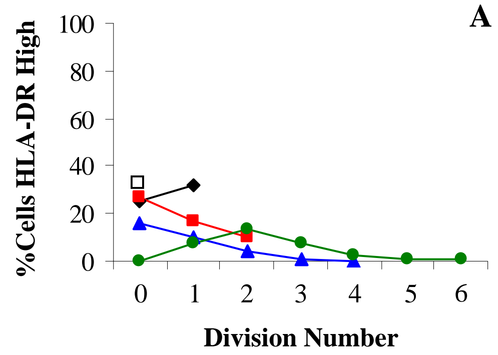
Differences in the profile of HLA-DR^{medium} expression were observed between CML patients. For example, variation was observed in the percentage of freshly isolated cells that expressed HLA-DR^{medium}, with 64% and 8% of cells from CMLs A and B expressing HLA-DR^{medium} respectively. In addition, differences were observed in the effect of culture

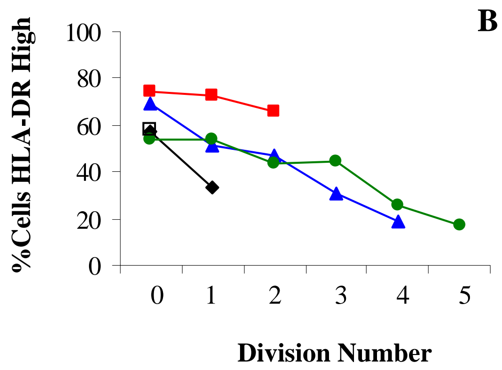
Figure 3.6B Changes in the Percentage of Cells Expressing HLA-DR^{High} with Cell Division & Culture Duration in Normal Donors and CML Patients.

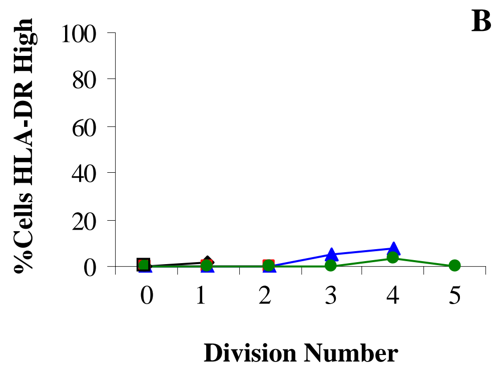
CD34^{+} cells were isolated from the bone marrow, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for HLA-DR expression using flow cytometry. The percentage of cells expressing high levels of HLA-DR in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. A decrease in the percentage of HLA-DR^{high} cells was observed with an increase in cell division number, but was unaffected by culture duration, in normal donors A, B and D. A decrease in the percentage of cells expressing high levels of HLA-DR was observed with an increase in culture duration in CML patient A, such that by day 4, virtually no cells expressing HLA-DR^{high} were observed. No cells expressing high levels of HLA-DR were observed in CML donors B and C over the 4 days of culture.

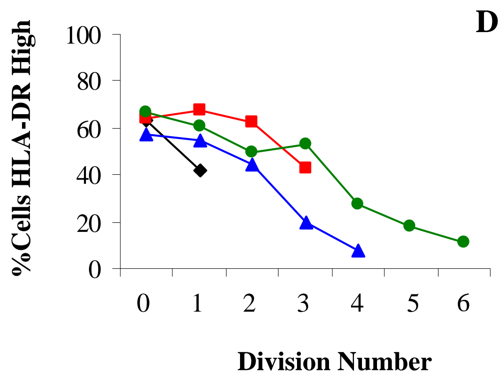
Normal Donors | **CML Patients**











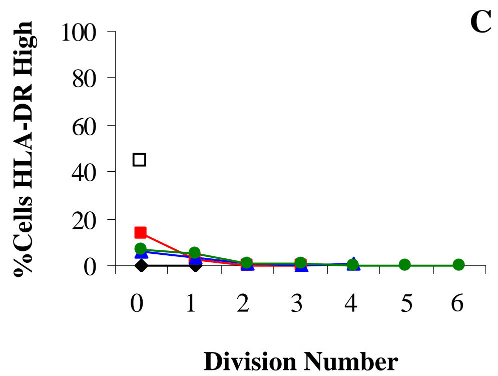
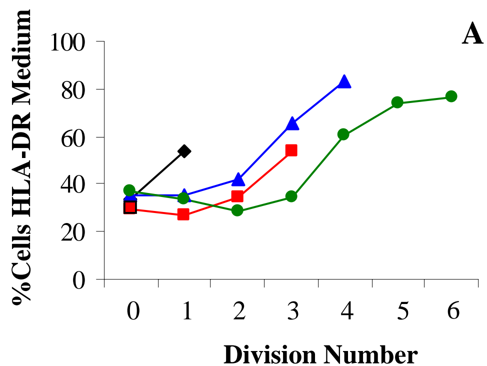


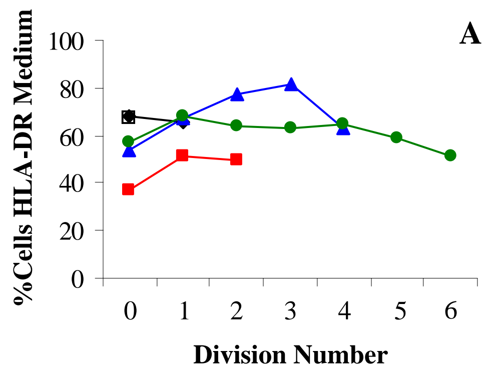
Figure 3.6C Changes in the Percentage of Cells Expressing HLA-DR^{Medium} with Cell Division & Culture Duration in Normal Donors and CML Patients.

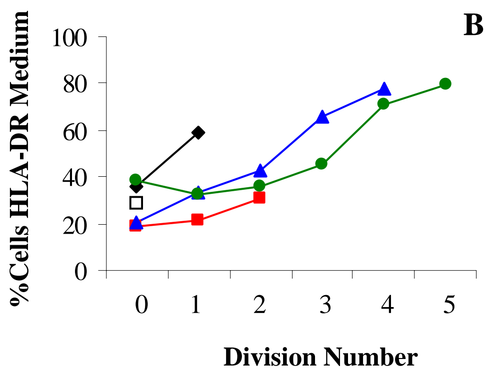
CD34^{+} cells were isolated from the peripheral blood, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for HLA-DR expression using flow cytometry. The percentage of cells expressing medium levels of HLA-DR in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. An increase in the percentage of cells expressing medium levels of HLA-DR was observed with an increase in cell division number in normal donors A, B and D. Marked variation in the effect of culture duration and/or cell division on the percentage of cells expressing HLA-DR^{medium} was observed in CML patients A, B and C.

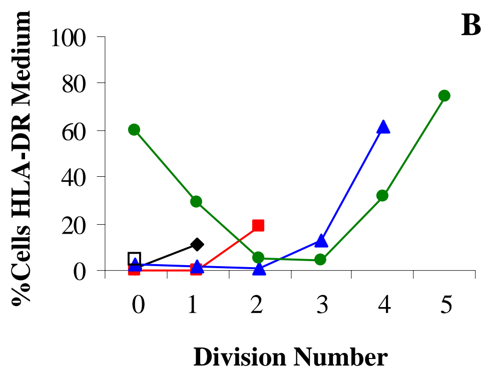
Normal Donors

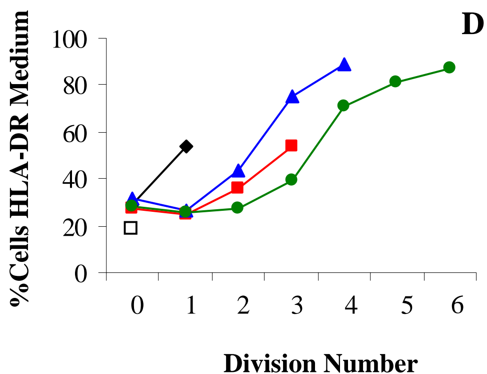
CML Patients

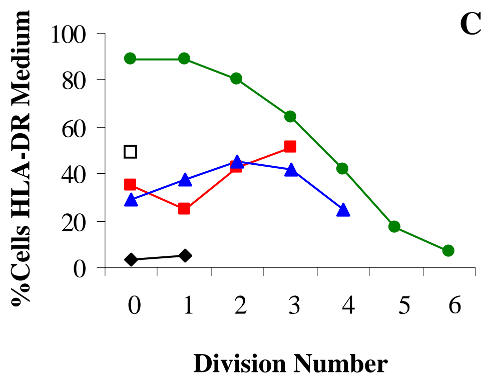












duration and cell division on the percentage of cells expressing HLA-DR^{medium}. In CML A, the percentage of cells expressing HLA-DR^{medium} was unchanged with cell division or culture duration. In CMLs B and C the percentage of cells expressing HLA-DR^{medium} increased with culture duration and was also modified by cell division, although the effect of cell division on the percentage of cells expressing HLA-DR^{medium} was unique to each patient. In CML B, the percentage of cells expressing HLA-DR^{medium} on day 4 of culture decreased from 60% to 5% over divisions 1 and 2, and subsequently increased to 75% by division 5 (Figure 3.6C). In CML C, the percentage of undivided cells expressing HLA-DR^{medium} increased from 35% on day 2 of culture to 89% on day 4 of culture, and subsequently decreased with cell division on day 4 of culture until 7% of cells expressed HLA-DR^{medium} in division 6.

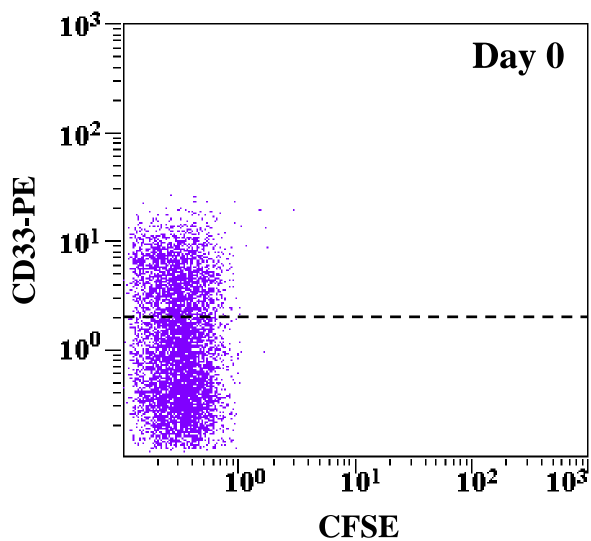
A comparison of the profile of HLA-DR^{medium} expression between normal donors and CML patients identified 3 major differences. Firstly, variations occurred in the percentage of undivided cells expressing HLA-DR^{medium} in CML patients but not in normal donors, with a greater percentage of cells from CML A expressing HLA-DR^{medium} and a lower percentage of cells from CML B expressing HLA-DR^{medium} on days 0-3 compared to normal donors. Secondly, the percentage of cells expressing HLA-DR^{medium} in CML patients was affected by culture duration, whereas normal donors were not. Finally, in normal donors $80 \pm 2\%$ of cells expressed HLA-DR^{medium} in division 6, whereas in CML patients this value ranged from 7% in CML C to 75% in CML B (Figure 3.6C).

3.2.2.5 CD33 Expression

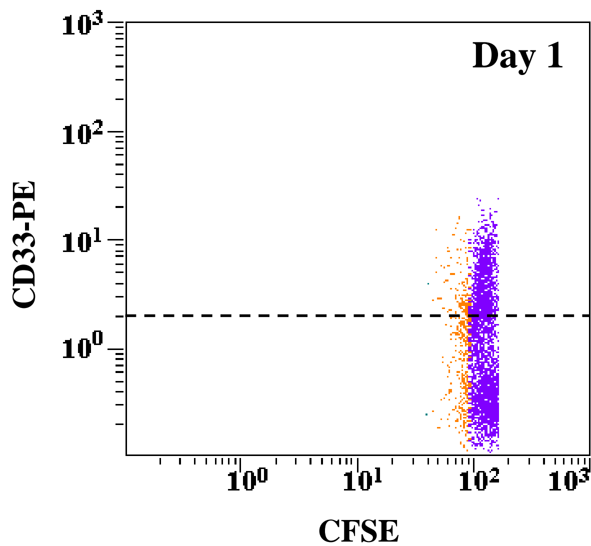
An example of changes in CD33 expression levels with cell division and culture duration for normal donor A is displayed in Figure 3.7A. Populations of $CD33^+$ and $CD33^-$ cells were present in freshly isolated samples and these positive and negative populations were maintained over 1 day of culture. On day 2 of culture there was an increase in the level of CD33 expression that was associated with cell division and this was most marked in divisions 2 and 3 where the MFI increased from approximately 7 units in undivided cells to approximately 12 units (Figure 3.7A). On days 3 and 4 of culture, discrete $CD33^+$ populations were present within each cell division and, as observed on day 2 of culture, there was an increase in the level of CD33 expression with cell division (MFI=8 on

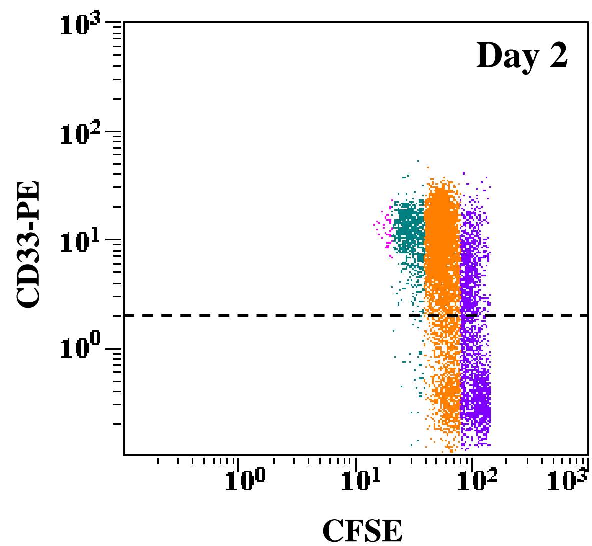
Figure 3.7A Change in CD33 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

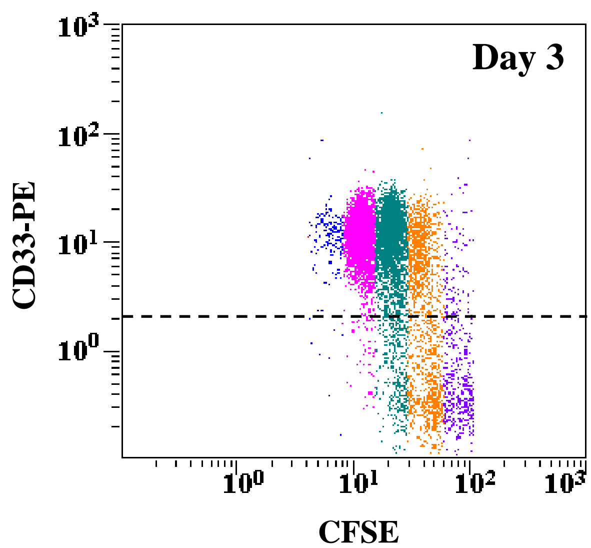
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD33 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD33, with the dashed line delineating cells that were positive and negative for CD33 expression, and the coloured dots distinguishing cell division number. Cells underwent 5 rounds of cell division over the 4 days of culture. Populations of CD33$^+$ and CD33$^-$ cells were present on day 0 and these populations were maintained over 1 day of culture. On day 2 of culture, an increase in the level of CD33 expression that was associated with cell division was observed after division 2, and was maintained over the duration of the culture. Results represent an example of flow cytometric data from normal donor A.

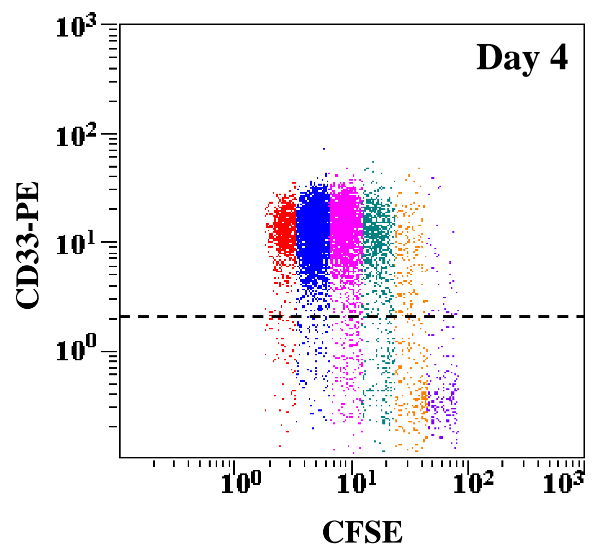

Day 0
CD33-PE
CFSE
10^0
10^1
10^2
10^3

Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6


Day 1
CD33-PE
CFSE


Day 2
CD33-PE
CFSE


Day 3
CD33-PE
CFSE


Day 4
CD33-PE
CFSE

undivided cells, MFI=15 in division 3). Virtually no CD33$^-$ cells were present after division 1 on day 2 of culture (Figure 3.7A).

Analysis of the percentage of cells expressing CD33 in normal donors over 4 days of culture demonstrated variations with cell division but not culture duration (Figure 3.7B). The pattern of CD33 expression was distinctly different for the 2 donors, however. For example, in Normal A the percentage of CD33$^+$ cells increased from 38$\pm$5.7 in undivided cells to 96% in division 5. In Normal C, the percentage of CD33$^+$ cells decreased from 97% in division 0 to 72% in division 2 on day 4 of culture, and subsequently increased to 94$\pm$1.5% over divisions 3-5 (Figure 3.7B).

The profile for the percentage of CD33$^+$ cells over 4 days of culture and 5 division cycles varied between CML patients (Figure 3.7B). In CMLs A and C the percentage of cells expressing CD33 increased with culture duration as well as cell division. In CML B, the percentage of CD33$^+$ cells was unaffected by culture duration and cell division over divisions 0-3 (90$\pm$2.6%), but decreased to 65% and 50% in divisions 4 and 5 respectively on day 4 of culture (Figure 3.7B). Variation in the percentage of starting cells that expressed CD33 was also observed between CML patients and ranged from 33% in CML A to 98% in CML B.

Excluding an increase in the percentage of cells expressing CD33 on days 1 and 2 of culture in CML A, the profile for CD33 expression in CML A was similar to Normal A (Figure 3.7B). Likewise, the CD33 expression profile observed in CML B was similar to that of Normal C. Although the profile for CML C was distinctly different to either Normal A or Normal C due to variations related to culture duration, the percentage of cells expressing CD33 in divisions 5 and 6 (100%) was similar to that observed in the normal donors (Figure 3.7B).

3.2.2.6 CD13 Expression

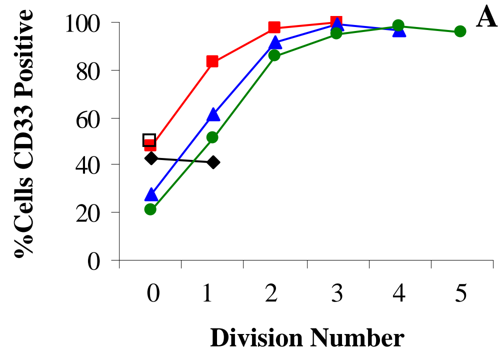
Figure 3.8A shows changes in CD13 expression levels with cell division over 4 days of culture for normal donor A. Similar to CD33 expression, populations of cells that were positive and negative for CD13 were present in freshly isolated cell suspensions. These

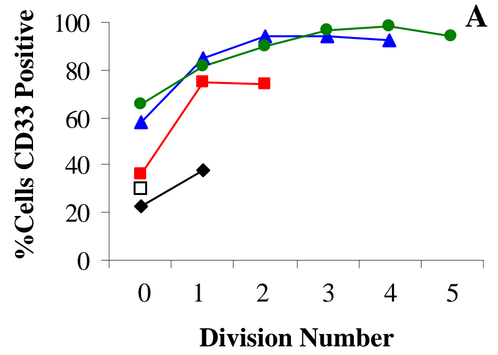
Figure 3.7B Changes in the Percentage of Cells Expressing CD33 with Cell Division & Culture Duration in Normal Donors and CML Patients.

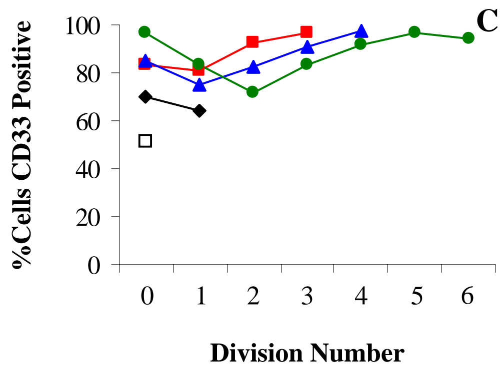
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD33 expression using flow cytometry. The percentage of cells that were positive for CD33 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 2 normal donors and 3 CML patients are displayed. Changes in the percentage of CD33 positive cells that were associated with cell division number and not length of time in culture were seen in normal donors A and C, and CML patient B. An increase in the percentage of $CD33^+$ cells with cell division was also observed in CML patients A and C, and was accompanied by an increase in the percentage of $CD33^+$ cells with an increase in culture duration.

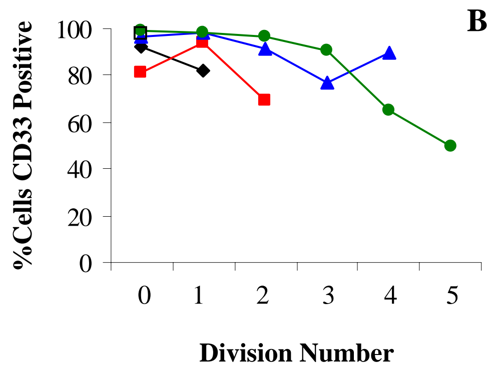
Normal Donors

CML Patients









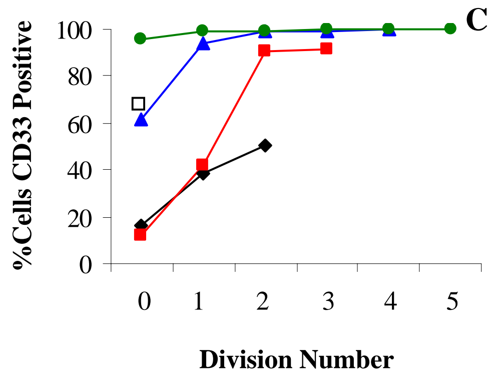
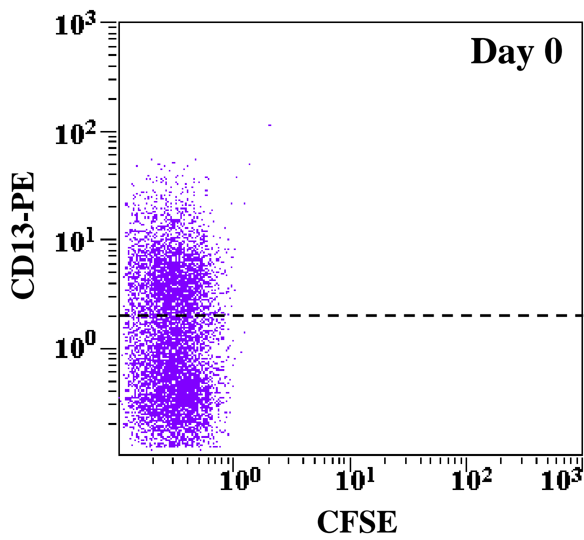
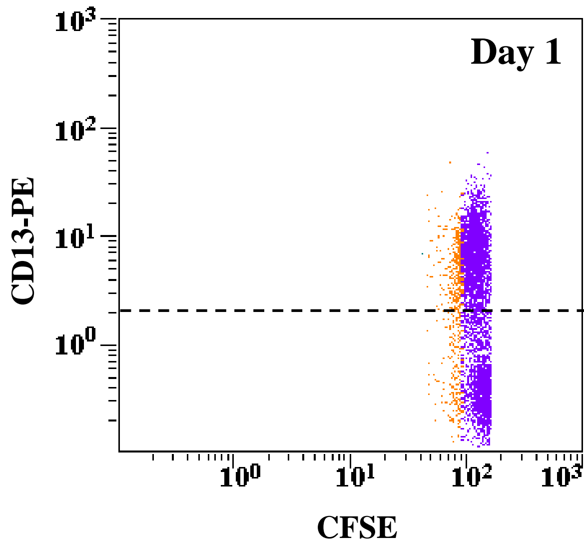


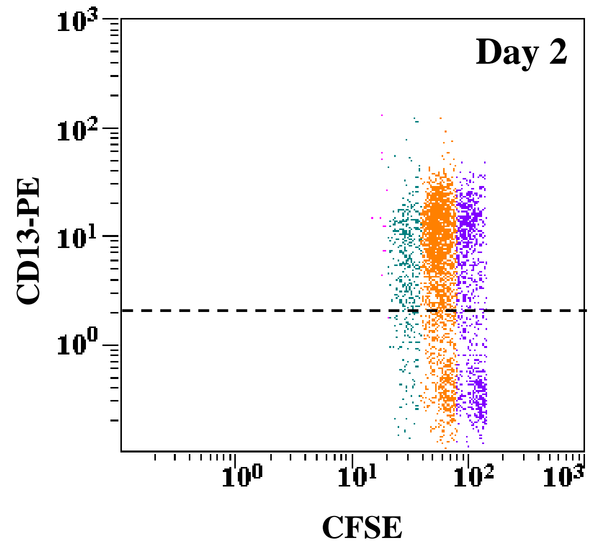
Figure 3.8A Change in CD13 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

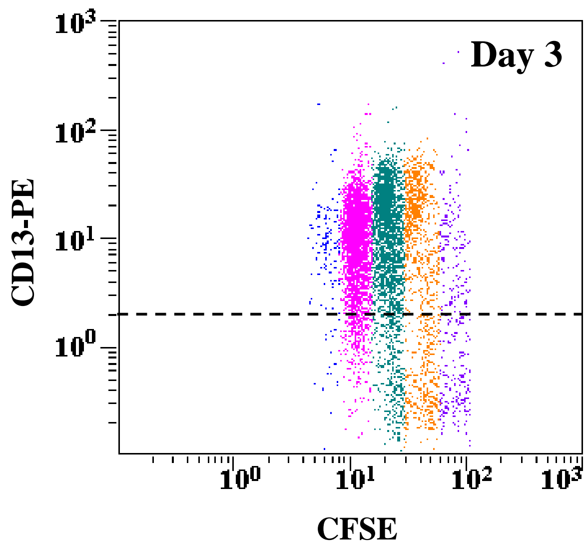
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD13 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD13, with the dashed line delineating cells that were positive and negative for CD13 expression, and the coloured dots distinguishing cell division number. Cells underwent 5 rounds of cell division over the 4 days of culture. Cells that were negative and positive for CD13 expression were observed on day 0, and were maintained over 2 days of culture and 2 division cycles. On day 3 of culture, the mean fluorescence intensity (MFI) of the undivided population increased and was maintained at this higher level across the 4 division cycles. On day 4 of culture, the MFI of cells decreased over divisions 3-5. Results represent an example of flow cytometric data from normal donor A.

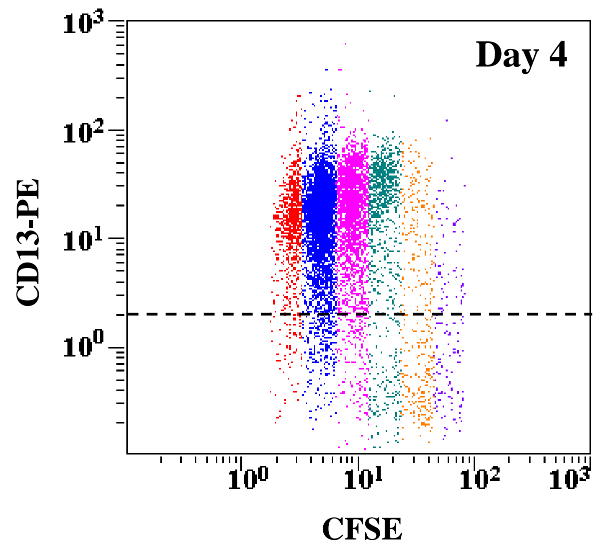


Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6









populations were still present after 2 days of culture and 2 division cycles, although the MFI of the positive population increased from 6 units on day 0 to 12 units on day 2 (Figure 3.8A). On day 3 of culture the MFI of the undivided positive population increased further to 18 units and CD13 expression was maintained at this higher level until divisions 3 and 4, where the MFI of the positive population decreased to 13 units. On day 4 of culture the MFI of the undivided positive population decreased to 10 units, but subsequently increased to 22 and 29 units over divisions 1 and 2 respectively. A decrease in CD13 expression again occurred over divisions 3-5, with the MFI of cells in division 5 measuring 20 units. Despite a decrease in the proportion of CD13$^-$ cells with culture, a CD13$^-$ population was present over the 4 days of culture and 5 division cycles (Figure 3.8A).

The profile for CD13 expression was similar in Normals A and D, with the percentage of CD13$^+$ cells increasing with cell division but unaffected by culture duration (Figure 3.8B). In addition, the percentage of cells expressing CD13 in division 0 remained stable at 57$\pm$3% in Normal A and 80$\pm$3% in Normal D over the 4 days of culture (Figure 3.8B).

The effect of cell division and culture duration on the percentage of CD13$^+$ cells was variable between each CML patient (Figure 3.8B). In CML A, the percentage of CD13$^+$ cells increased with an increase in culture duration, but was relatively unchanged with cell division over each day of culture. An increase in the percentage of CD13$^+$ cells with an increase in culture duration was similarly seen in CML B, although a concomitant decrease in the percentage of CD13$^+$ cells with cell division also occurred (Figure 3.8B). In CML C, a 300% increase in the percentage of CD13$^+$ cells was observed after division 1 on day 2 of culture, but was unchanged with further division across the culture period. The percentage of CD13$^+$ cells in CML C also increased from 20% on days 0-2 of culture to 90-100% on days 3 and 4 of culture (Figure 3.8B).

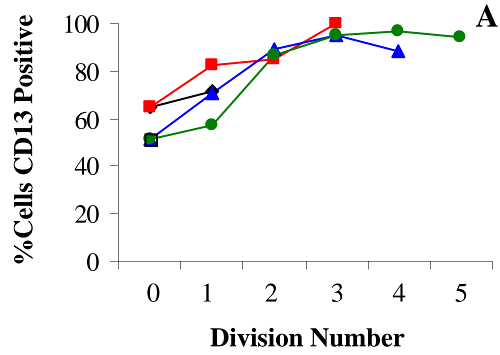
Although the profile for the percentage of cells expressing CD13 was conserved across normal donors, the profiles for CML patients were markedly different between patients as well as compared to normal donors (Figure 3.8B). In particular, cells from CML patients were affected to a greater degree by culture duration and an increase in the percentage of cells expressing CD13 with cell division was not observed. In spite of these differences,

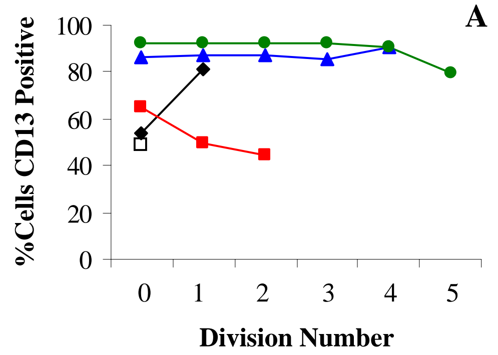
Figure 3.8B Changes in the Percentage of Cells Expressing CD13 with Cell Division & Culture Duration in Normal Donors and CML Patients.

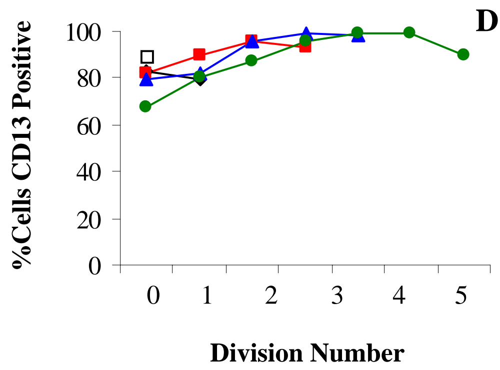
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD13 expression using flow cytometry. The percentage of cells that were positive for CD13 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 2 normal donors and 3 CML patients are displayed. In normal donors A and D, an increase in the percentage of $CD13^+$ cells that was related to cell division number, but not culture duration, was observed. The percentage of $CD13^+$ cells in CML donors increased with a corresponding increase in culture duration, and was unaffected by cell division in CML patient A. In CML patient B, the percentage of $CD13^+$ cells decreased with cell division on days 2-4 of culture, while in CML C, the percentage of $CD13^+$ cells increased with cell division on day 1 of culture, but was thereafter unaffected by cell division.

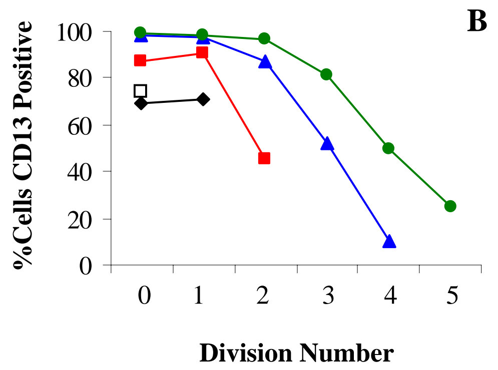
Normal Donors

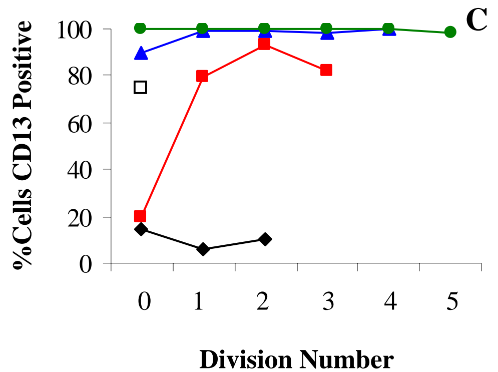
CML Patients











the percentage of cells expressing CD13 in division 5 was 80% in CML A and 98% in CML C, similar to the value observed in normal donors (average of 92%) (Figure 3.8B).

3.2.2.7 CD36 Expression

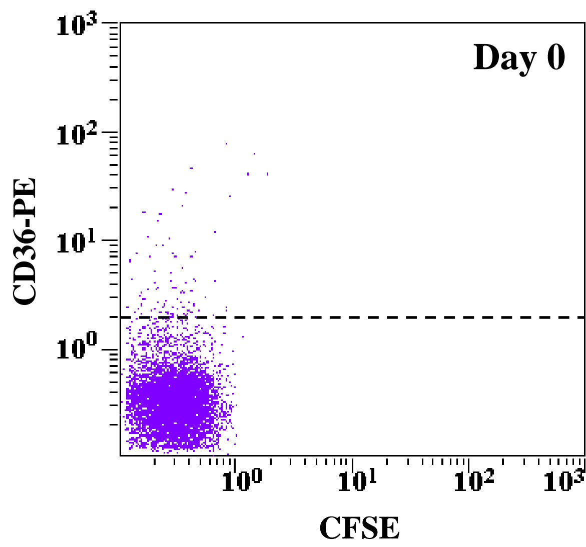
The profile of CD36 expression over 4 days of culture in normal donor A is shown in Figure 3.9A. CD36 expression was absent from most cells at the onset of the culture, however, after 1 day of culture a population of $CD36^+$ cells with a maximal fluorescence of approximately 30 units was present and remained over the 4 days of culture and 6 division cycles (Figure 3.9A). On days 1-3 of culture, a decrease in the level of CD36 expression was seen with an increase in cell division. For example, on day 3 of culture the MFI of undivided cells was approximately 20 units and the MFI of cells in division 4 was approximately 5 units. On day 4 of culture the level of CD36 expression was unchanged with cell division (Figure 3.9A).

The effect of cell division on the percentage of $CD36^+$ cells was conserved across all normal donors and was associated with a decrease in the percentage of $CD36^+$ cells over divisions 0-3 (Figure 3.9B). In divisions 5 and 6, the percentage of $CD36^+$ cells subsequently increased to 60-70% in all normal donors (Figure 3.9B). The effect of culture duration on CD36 expression, however, varied between normal donors. In Normals A and B, the percentage of $CD36^+$ cells in division 0 increased from 5% to 30% (Normal A) or 50% (Normal B) on day 2 of culture, but was thereafter unaffected by culture duration (Figure 3.9B). Although an increase in the percentage of $CD36^+$ cells in division 0 was also observed with culture duration in Normal C, this was more marked than in Normals A and B and occurred over each of the 4 days of culture. For example, approximately 5% of freshly isolated cells from Normal C were $CD36^+$ and by day 4, 90% of undivided cells were $CD36^+$ (Figure 3.9B).

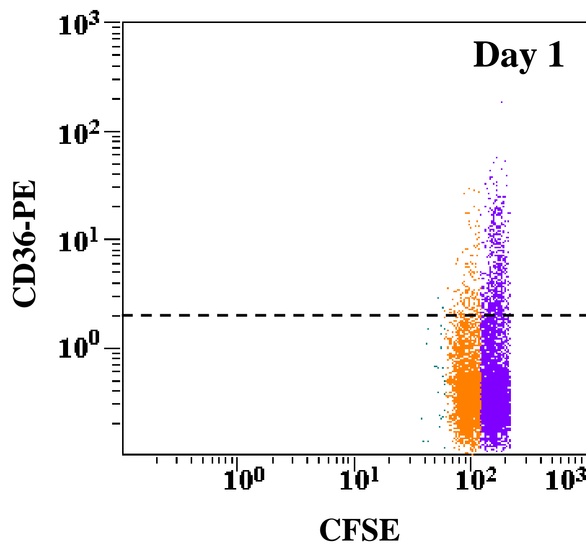
In CML patients, different trends in CD36 expression were observed (Figure 3.9B). Variation in the percentage of $CD36^+$ cells was evident in freshly isolated cells and ranged from 5% in CML B to 34% in CML C (Figure 3.9B). A 300-500% increase in the percentage of undivided cells expressing CD36 was observed in each CML patient on day 4 of culture, although the effect of division on the percentage of cells expressing CD36 was

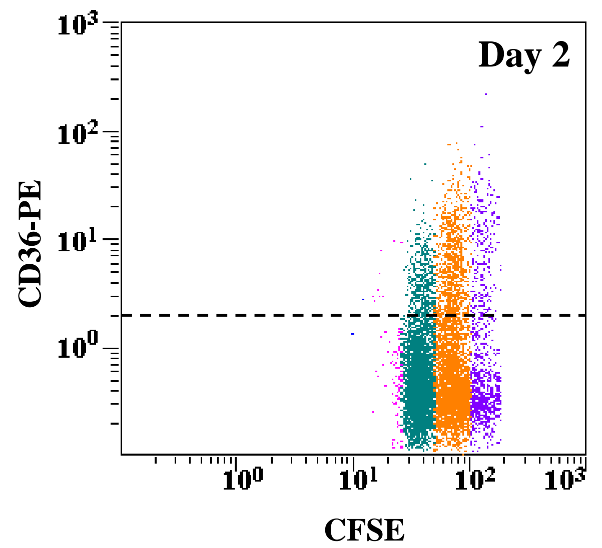
Figure 3.9A Change in CD36 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

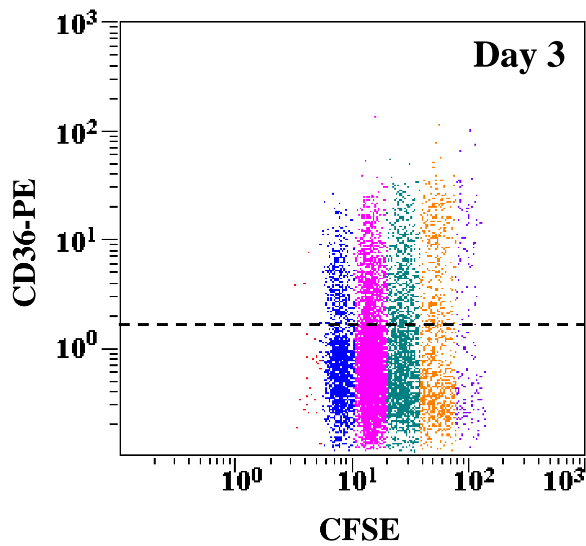
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD36 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD36, with the dashed line delineating cells that were positive and negative for CD36 expression, and the coloured dots distinguishing cell division number. Cells underwent 6 rounds of cell division over the 4 days of culture. A small population of cells expressing CD36 was observed in freshly isolated cells, and was maintained over the 4 days of culture and 6 division cycles. On days 1-3 of culture, a decrease in the level of CD36 expression was observed with an increase in cell division. On day 4 of culture the mean fluorescence intensity (MFI) of cells expressing CD36 was unchanged with cell division. Results represent an example of flow cytometric data from normal donor A.



Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6







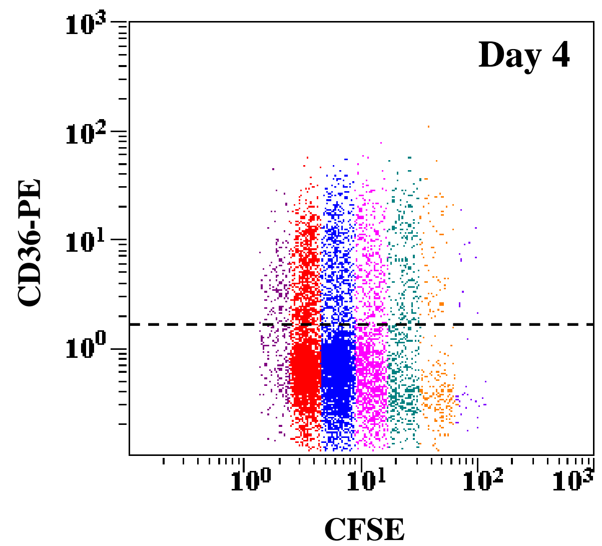
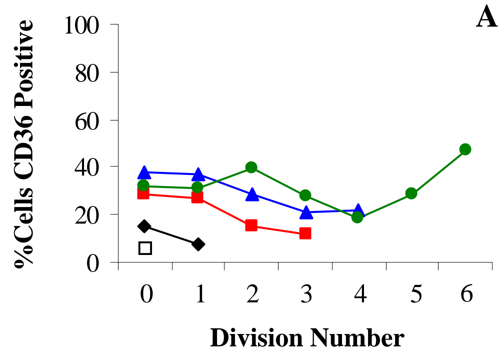
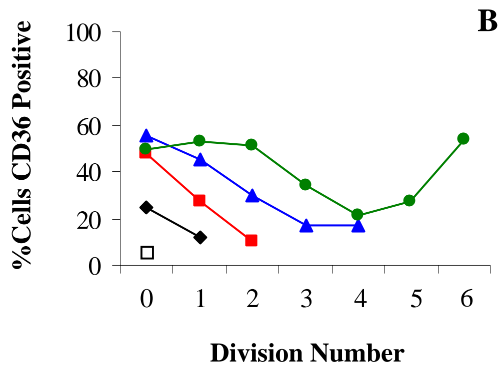


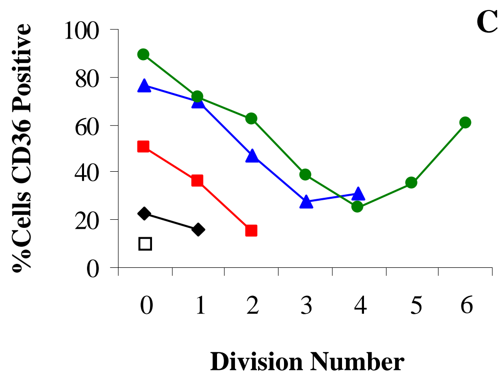
Figure 3.9B Changes in the Percentage of Cells Expressing CD36 with Cell Division & Culture Duration in Normal Donors and CML Patients.

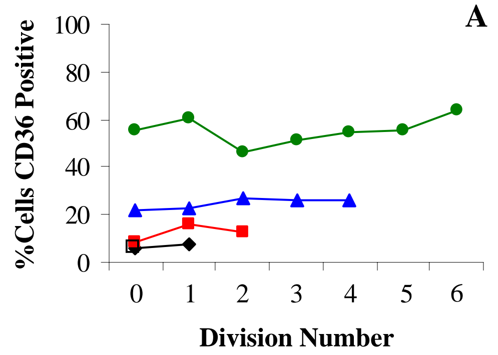
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD36 expression using flow cytometry. The percentage of cells that were positive for CD36 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. In normal donors A, B and C, an increase in the percentage of cells that expressed CD36 was observed with an increase in culture duration, but decreased with a corresponding increase in cell division number. This occurred until division number 4, when the percentage of cells expressing CD36 increased with cell division. In CML patient A, CD36 expression increased with culture duration but was unaffected by cell division. An increase in the percentage of cells expressing CD36 occurred with an increase in cell division but not culture duration on days 1-3 for CML patients B and C. On day 4 of culture there was an increase in the percentage of undivided cells expressing CD36 in CML patients B and C, relative to days 1-3 of culture.

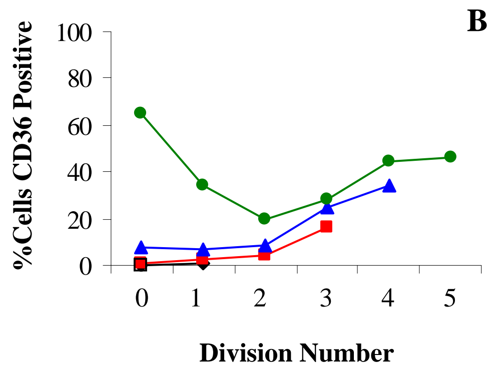
Normal Donors | **CML Patients**

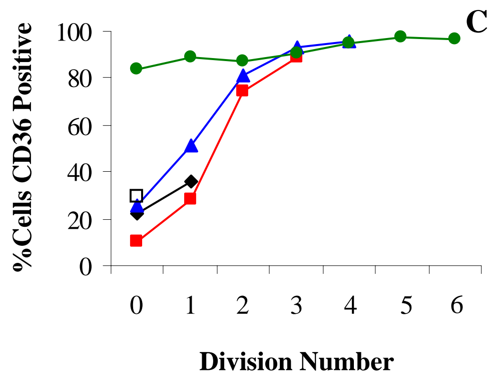












variable. In CML A, the percentage of CD36+ cells was relatively unaffected by cell division number within each day of culture. In CML C, an increase in the percentage of CD36+ cells was observed with an increase in cell division number on culture days 1-3, while no cell division associated change in CD36 expression was observed on day 4 of culture (Figure 3.9B).

The greatest difference in CD36 expression profiles between normal donors and CML patients regarded variation in expression with cell division (Figure 3.9B). In normal donors, changes in the percentage of CD36+ cells with cell division were uniform, whereas the effect of cell division on the percentage of cells expressing CD36 in CML patients was variable. In addition, the percentage of CD36+ cells in division 5 was conserved at 48$\pm$10% in normal donors, but was elevated to 99% in CML C (Figure 3.9B).

3.2.2.8 CD14 Expression

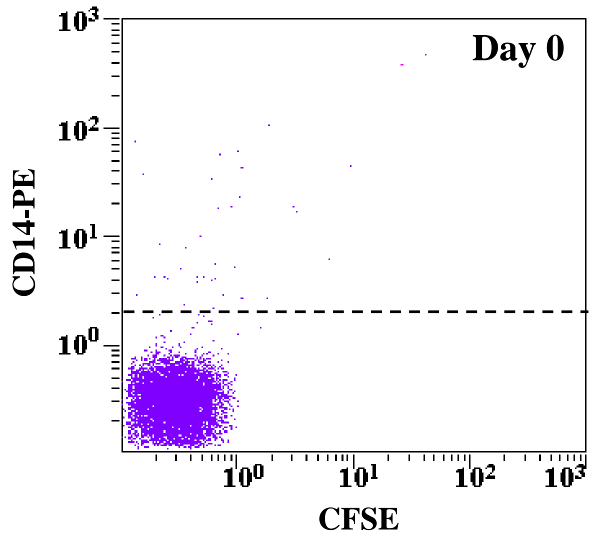
Figure 3.10A shows changes in the level of CD14 expression with cell division over 4 days of culture for Normal A. At the initiation of culture, virtually no cells expressed CD14. A CD14+ population was observed as the cells entered higher rounds of cell division, particularly once the cells entered division 3 on days 3 and 4 of culture. Variation in the level of CD14 expression was observed within the CD14+ population, with an even spread of cells expressing low to high levels of CD14. While an increase in the proportion of cells expressing CD14 was observed with an increase in cell division, the level of CD14 expression within each division was unchanged (Figure 3.10A).

In Figure 3.10B, a similar trend for the percentage of cells expressing CD14 was observed in all normal donors. An increase in the percentage of CD14+ cells was observed in division numbers 4 and 5 in Normals A and D and approximately 10% of cells expressed CD14 on day 4 of culture across all division cycles in Normal E (Figure 3.10B).

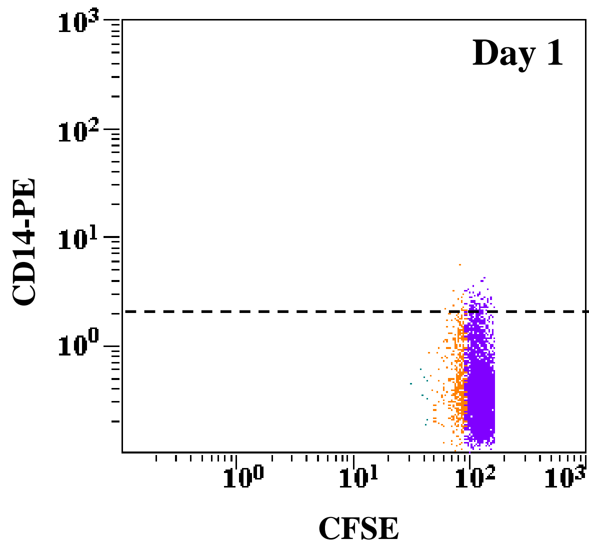
Variation was observed in the profile of CD14 expression in CML patients (Figure 3.10B). Virtually no CD14 expression was observed for CML A across the culture period. In CML B and C 57% and 89% of cells respectively were CD14+ on day 4 in division 0 and this percentage decreased with a concomitant increase in cell division number such that 1-6%

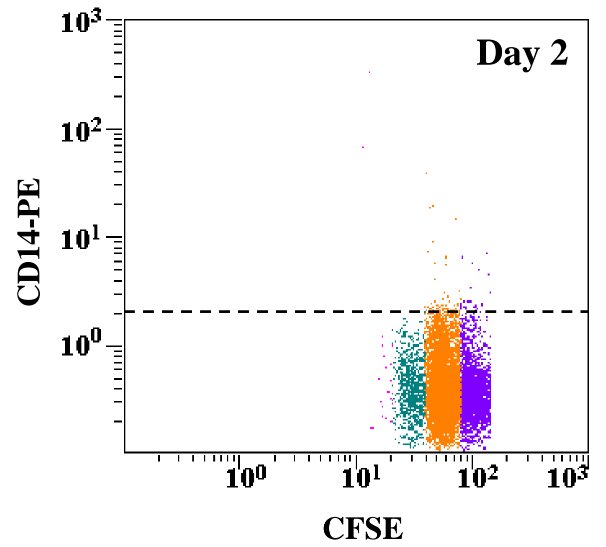
Figure 3.10A Change in CD14 Expression with Cell Division on $CD34^+$ Cells from Normal Donor A.

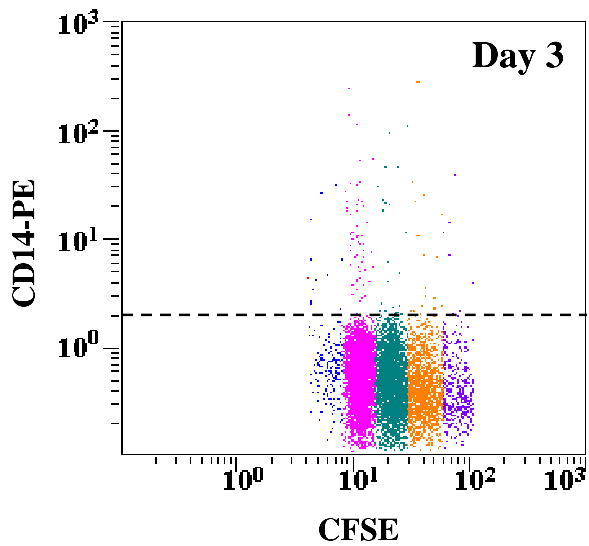
$CD34^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD14 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD14, with the dashed line delineating cells that were positive and negative for CD14 expression, and the coloured dots distinguishing cell division number. Cells underwent 5 rounds of cell division over the 4 days of culture. Although few freshly isolated cells expressed CD14, a marked increase in the percentage of $CD14^+$ cells was observed after division 2. The level of CD14 expression was not affected by subsequent cell division or culture duration. Results represent an example of flow cytometric data from normal donor A.


Day 0
CD14-PE
CFSE
10^0
10^1
10^2
10^3

Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6


Day 1
CD14-PE
CFSE


Day 2
CD14-PE
CFSE


Day 3
CD14-PE
CFSE

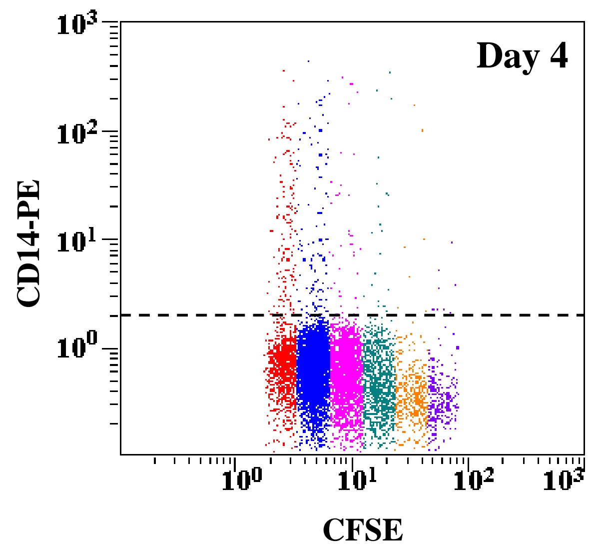
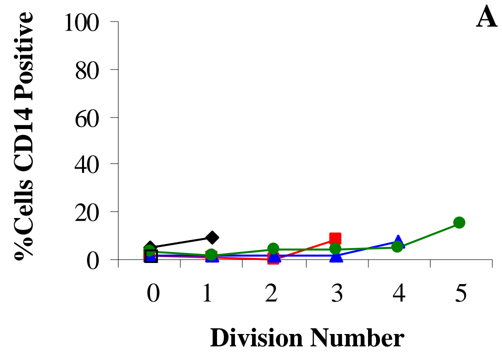

Day 4
CD14-PE
CFSE

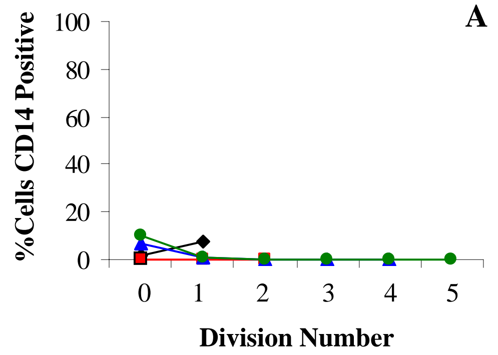
Figure 3.10B Changes in the Percentage of Cells Expressing CD14 with Cell Division & Culture Duration in Normal Donors and CML Patients.

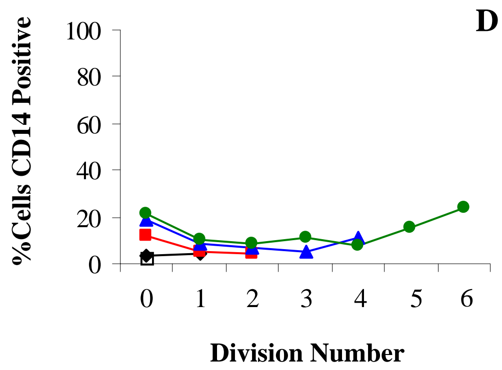
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD14 expression using flow cytometry. The percentage of cells that were positive for CD14 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. An increase in the percentage of $CD14^+$ cells was observed after cell division 3 in normal donors A and D, while expression was consistent in normal donor E. No CD14 expression was observed in CML patient A. An increase in the percentage of $CD14^+$ cells in CML patients B and C was seen in division 0 on day 4 of culture and this expression decreased with a corresponding increase in cell division number.

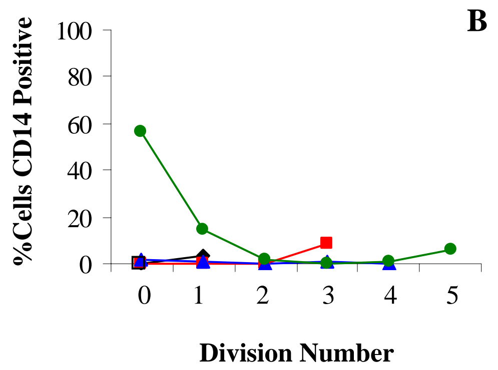
Normal Donors

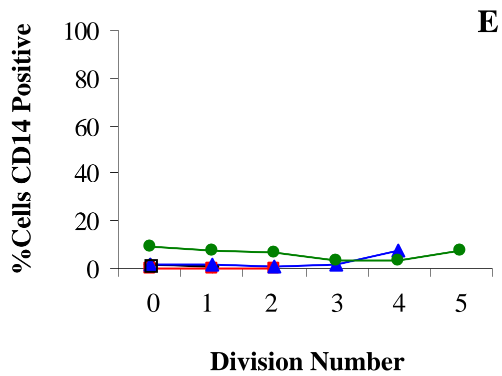
CML Patients

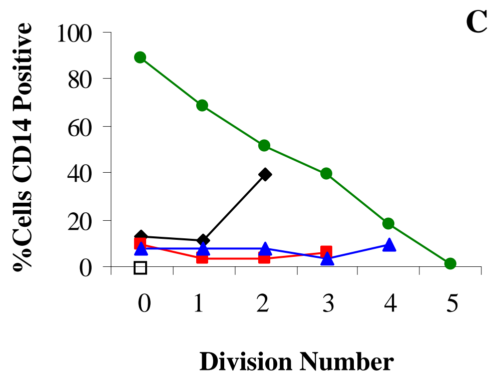












of cells were CD14$^+$ in division 5 (Figure 3.10B).

A comparison of the profile of CD14 expression for normal donors and CML patients identified that although the percentage of starting cells that expressed CD14 was uniform across all normal donors and CML patients, profound differences were observed on day 4 of culture (Figure 3.10B). This was related to an increase in the percentage of undivided cells expressing CD14 on day 4 of culture in CMLs B and C. In addition, the percentage of cells expressing CD14 after division 4 did not increase in CMLs A and C.

3.2.2.9 CD11c Expression

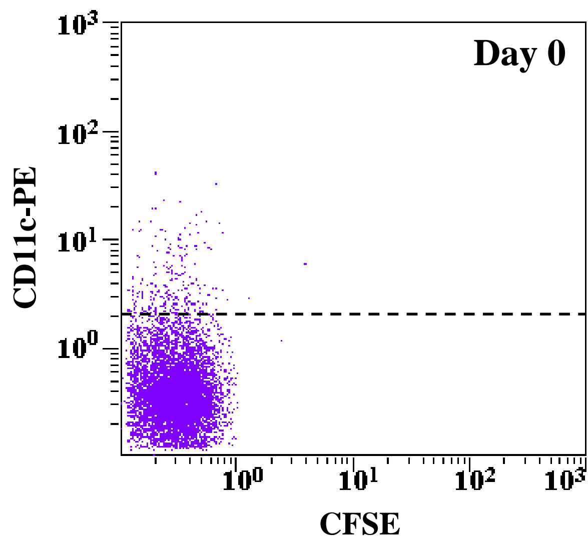
Figure 3.11A displays CD11c expression by CFSE labelled cells from normal donor A over 4 days of culture. A small population of freshly isolated cells expressed CD11c and this population was maintained over 4 days of culture and 5 division cycles. The level of CD11c expression that was observed on freshly isolated cells did not change over the 4 days and 5 division cycles, indicating that cell division and culture duration had no effect on CD11c expression levels by these cells.

Differences in the percentage of cells expressing CD11c were apparent between normal donors over 4 days of culture (Figure 3.11B). In Normals A and B, the percentage of cells expressing CD11c was relatively unchanged by cell division or culture duration. In Normal C, an increase in the percentage of CD11c$^+$ cells was observed with an increase in culture duration. For example, the percentage of CD11c$^+$ cells in division 0 increased from 25% on day 1 of culture to 80% on day 4 of culture (Figure 3.11B). Concomitant with an increase in CD11c expression in division 0 over the 4 days in culture in Normal C, was a decrease in the percentage of CD11c$^+$ cells with cell division (Figure 3.11B).

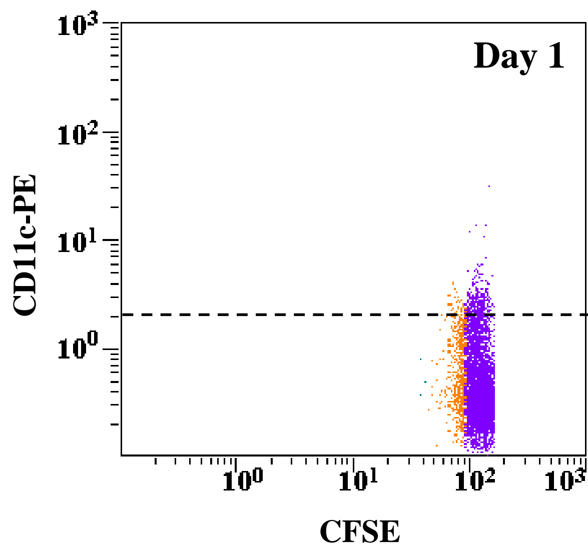
Different patterns of CD11c expression were also observed for cells from CML patients, with approximately 5% of cells from CML A expressing CD11c over the 4 days in culture (Figure 3.11B). CML B did not express CD11c on days 1-3, but on day 4 of culture 60% of cells were CD11c$^+$. This percentage decreased to 15% at division 1 and remained at 0% until division 4 where 20% of cells were CD11c$^+$. A similar result was observed for CML C, with 80% of cells in division 0 expressing CD11c on day 4 of culture. The percentage

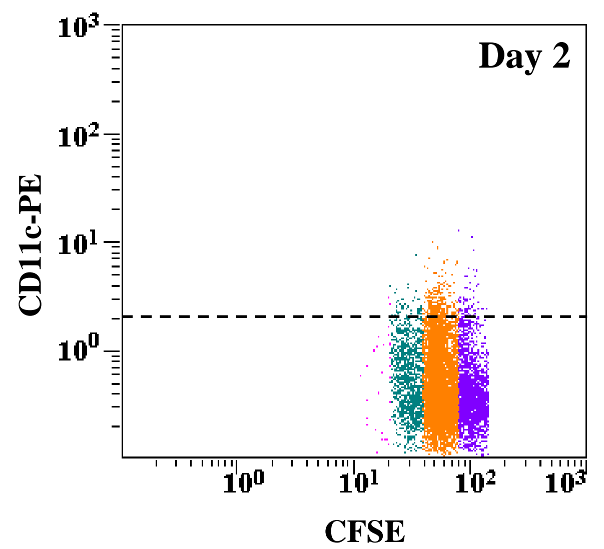
Figure 3.11A Change in CD11c Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

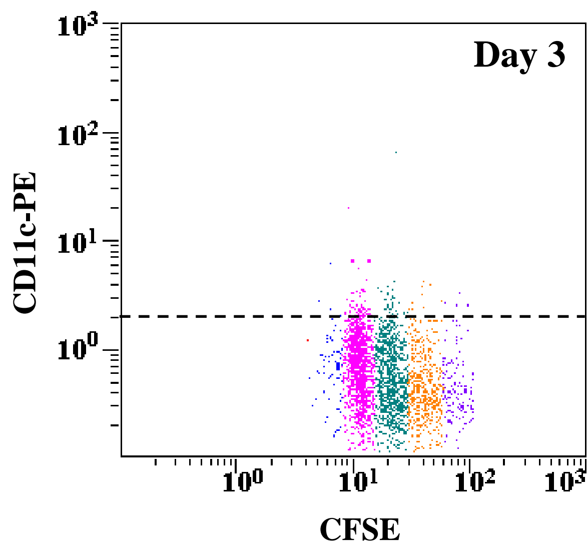
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD11c expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD11c, with the dashed line delineating cells that were positive and negative for CD11c expression, and the coloured dots distinguishing cell division number. Cells underwent 5 rounds of cell division over the 4 days of culture. A small population of cells expressing CD11c were observed in freshly isolated cells, and this population was maintained across the culture period. The level of CD11c expression was not affected by culture duration or cell division. Results represent an example of flow cytometric data from normal donor A.


Day 0
CD11c-PE
CFSE
10^0
10^1
10^2
10^3

Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6


Day 1
CD11c-PE
CFSE


Day 2
CD11c-PE
CFSE


Day 3
CD11c-PE
CFSE

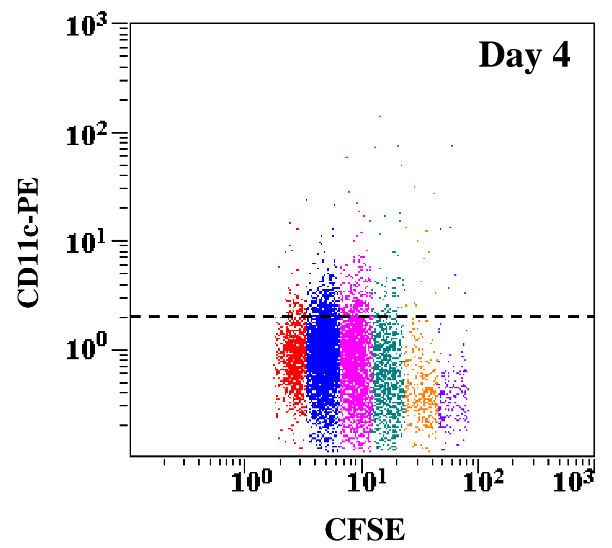
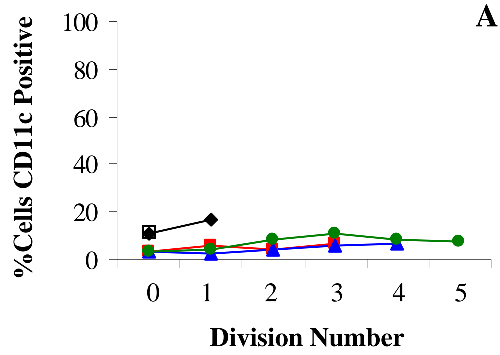

Day 4
CD11c-PE
CFSE

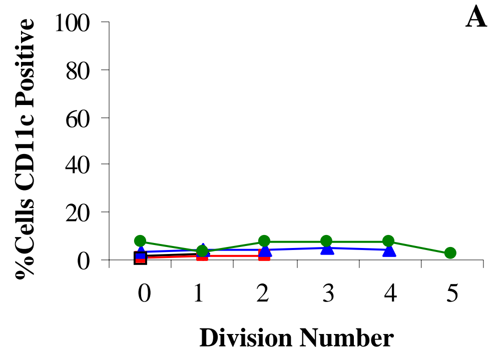
Figure 3.11B Changes in the Percentage of Cells Expressing CD11c with Cell Division & Culture Duration in Normal Donors and CML Patients.

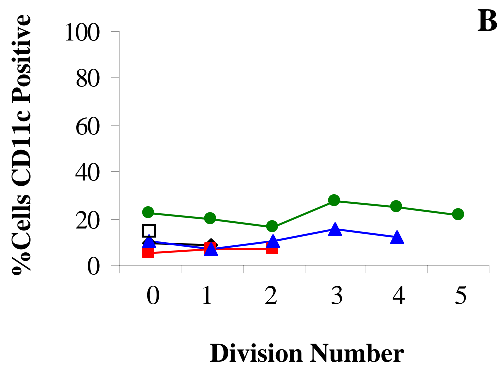
CD34$^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD11c expression using flow cytometry. The percentage of cells that were positive for CD11c expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. Marked variation was observed in the percentage of CD11c$^+$ cells in normal donors and CML patients. Low levels of CD11c$^+$ cells were observed in normal donor A and B across the culture period. An increase in the percentage of CD11c$^+$ cells was observed with an increase in culture duration in normal donor C and was accompanied by a concomitant decrease in the percentage of CD11c$^+$ cells with cell division. No CD11c$^+$ cells were observed in CML patient A. An increase in the percentage of CD11c$^+$ cells was observed in CML patients B and C on day 4 of culture and decreased with cell division.

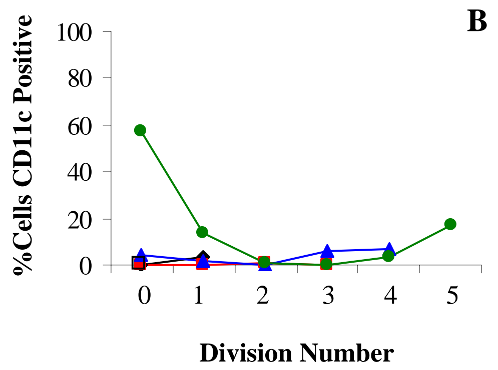
Normal Donors

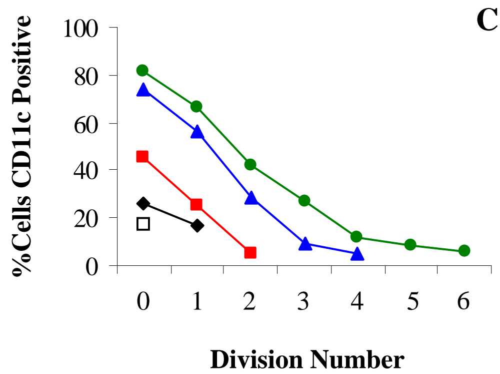
CML Patients

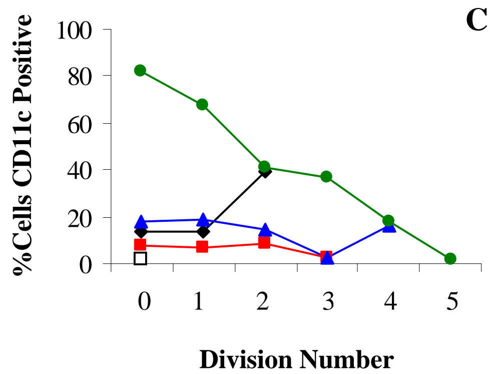












of cells expressing CD11c decreased with each successive division in CML C, and by division number 5, virtually no cells expressed CD11c (Figure 3.11B).

3.2.2.10 CD15 Expression

Figure 3.12A demonstrates that no CD15 expression was observed on freshly isolated cells or cells that had been cultured for 1-4 days from normal donor A. A small population of cells expressing low levels of CD15 were observed in division 4 on day 4 of culture, however this may be due to non-specific binding (Figure 3.12A). These results are illustrated graphically in Figure 3.12B, where no CD15$^+$ cells were detected in Normal A over 5 division cycles and 4 days of culture, excluding division 4 on day 4 of culture. Although no cells expressed CD15 on days 1-3 in Normal C, on day 4 of culture 18% of cells were CD15$^+$ in division 0 on day 4 of culture. This percentage decreased to 0% by division 2 (Figure 3.12B).

No CD15 expression was also observed on CD34$^+$ cells from CML A (Figure 3.12B). A population of cells that expressed CD15 in CMLs B and D was seen on days 3 and/or 4 of culture and this expression dropped rapidly over cell divisions 1 and 2 such that no CD15$^+$ cells were seen after division 1 (Figure 3.12B).

3.2.3 Effect of Cell Division on the Level of Surface Marker Expression

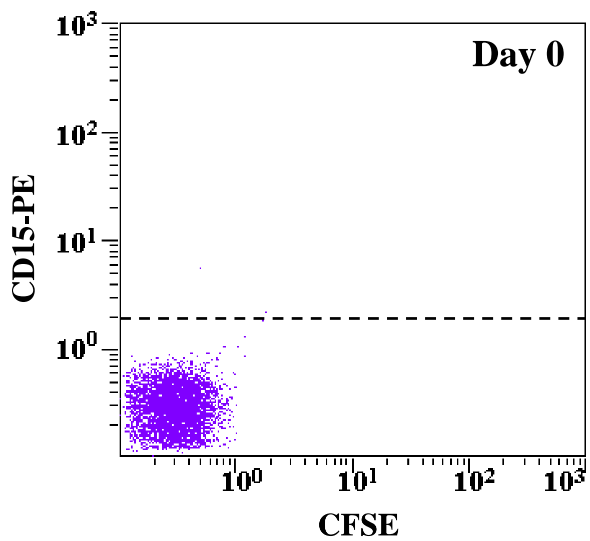
In section 3.2.2, the effect of cell division on the percentage of cells that were positive for various phenotypic markers was examined. In normal donors, the percentage of cells expressing CD38, HLA-DR, CD33 and CD13 appeared to be regulated by cell division alone and unaffected by culture duration. To determine whether variation existed in the level of expression of these proteins across normal donors and CML patients, and to examine changes in the level of expression of these proteins with culture duration and cell division, the mean fluorescence intensity (MFI) of the positive population was analysed with respect to cell division number over 4 days of culture.

3.2.3.1 Changes in the Level of CD38 Expression

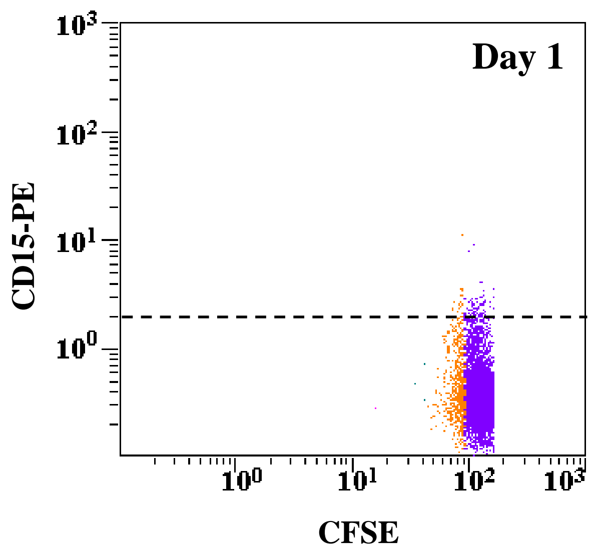
Changes in the level of CD38 expression with cell division are displayed in Figure 3.13. In normal donors, similar levels of CD38 expression were seen on cells over the 4 days of

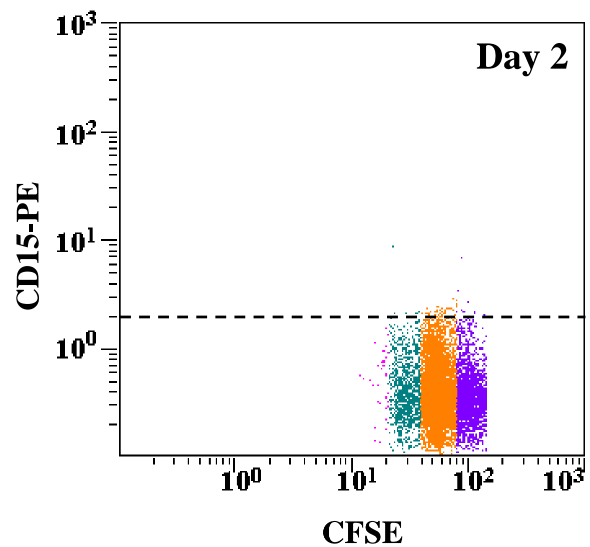
Figure 3.12A Change in CD15 Expression with Cell Division on CD34$^+$ Cells from Normal Donor A.

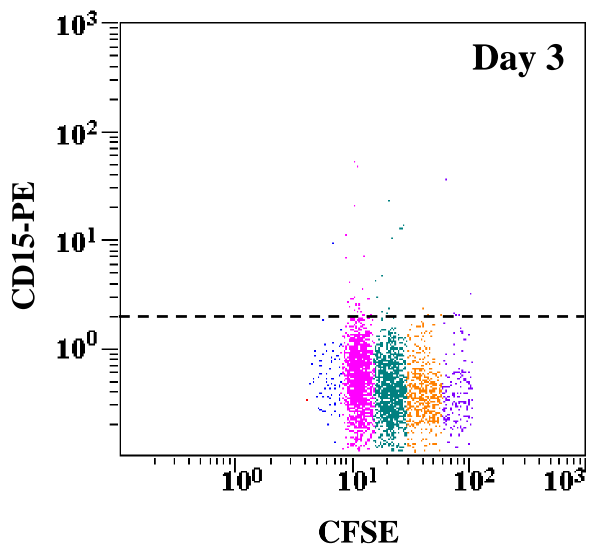
CD34$^+$ cells were isolated from the bone marrow of normal donors, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD15 expression using flow cytometry. Dot-plot analysis was used to examine the effect of cell division on the expression of CD15, with the dashed line delineating cells that were positive and negative for CD15 expression, and the coloured dots distinguishing cell division number. Cells underwent 5 rounds of cell division over the 4 days of culture. No cells expressed CD15 across the culture period. Results represent an example of flow cytometric data from normal donor A.


Day 0
CD15-PE
CFSE

Division 0
Division 1
Division 2
Division 3
Division 4
Division 5
Division 6


Day 1
CD15-PE
CFSE


Day 2
CD15-PE
CFSE


Day 3
CD15-PE
CFSE

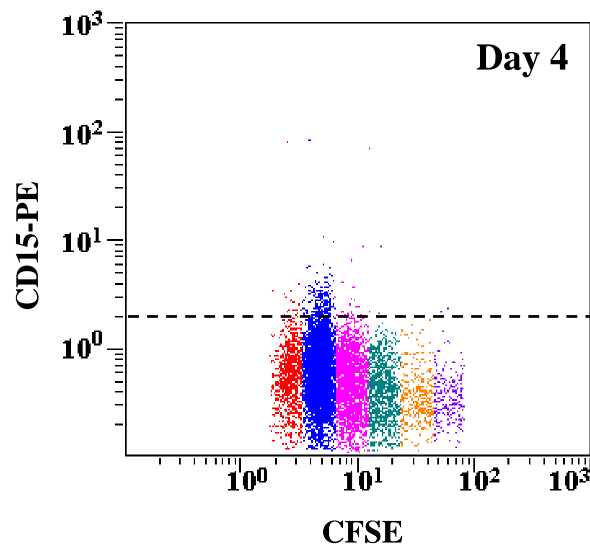
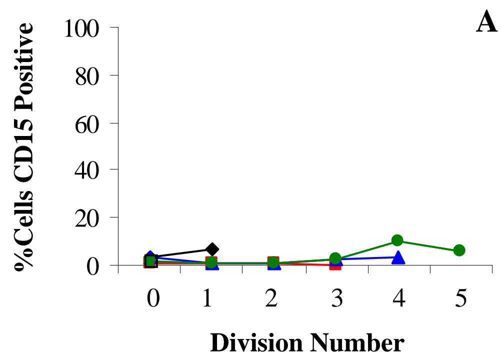

Day 4
CD15-PE
CFSE

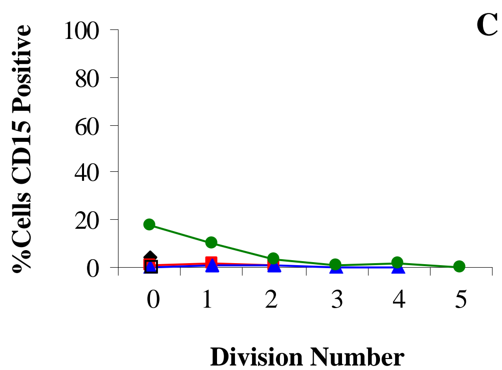
Figure 3.12B Changes in the Percentage of Cells Expressing CD15 with Cell Division & Culture Duration in Normal Donors and CML Patients.

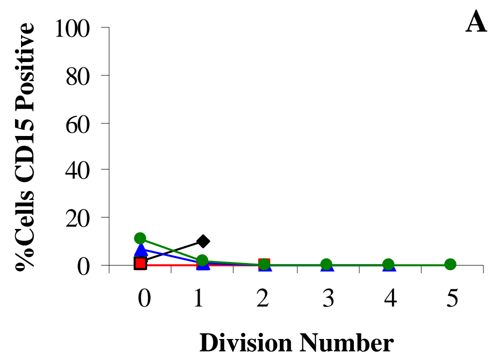
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD15 expression using flow cytometry. The percentage of cells that were positive for CD15 expression in each cell division was calculated, and plotted for each of the 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 2 normal donors and 3 CML patients are displayed. $CD15^+$ cells were not observed in normal donor A or CML patient A across the culture period. In normal donor C and CML patients B and D, an increase in the percentage of $CD15^+$ cells in division 0 was observed on days 3 and/or 4 of culture, with these cells losing expression by division number 2.

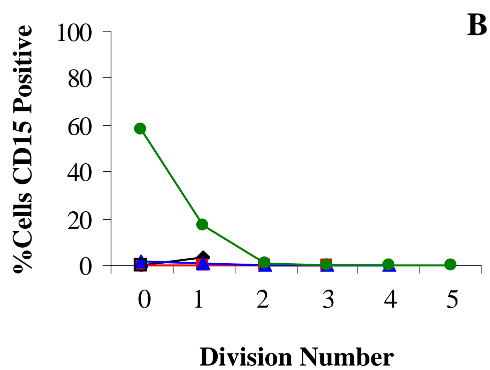
Normal Donors

CML Patients









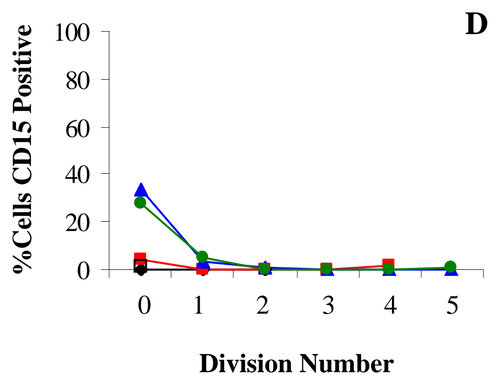
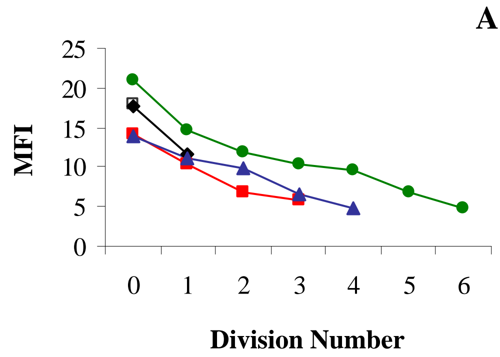


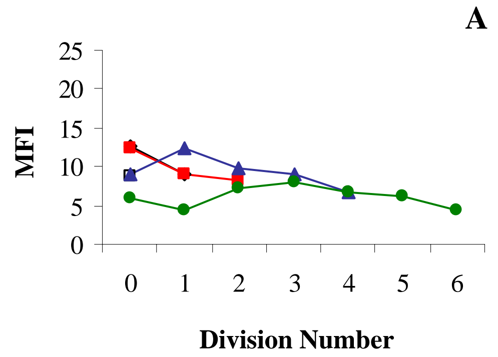
Figure 3.13 Analysis of CD38 Expression on Normal and CML $CD34^+$ Cells.

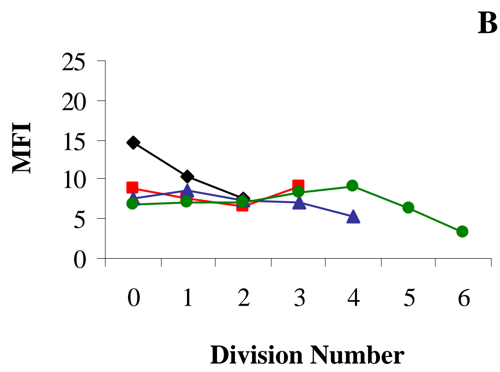
$CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD38 expression using flow cytometry. The mean fluorescence intensity (MFI) of cells that were positive for CD38 expression in each cell division was analysed, and plotted over 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. In normal donor A, the level of CD38 expression decreased with cell division, but was not affected by culture duration. In normal donors B and C the level of CD38 expression was unchanged with cell division and culture duration, excluding day 1 of culture for normal donor B and day 0 of culture for normal donor C. In CML patients A and B, the level of CD38 expression was relatively unaffected by culture duration and cell division. In CML patient C, the level of CD38 expression decreased with an increase in cell division.

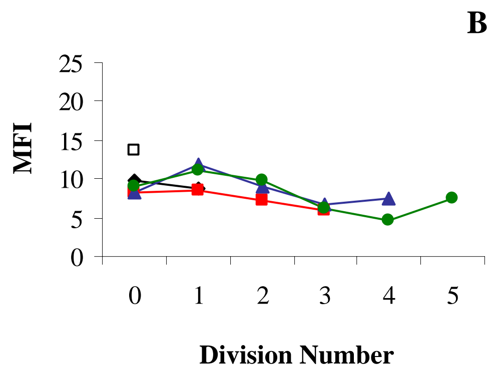
Normal Donors

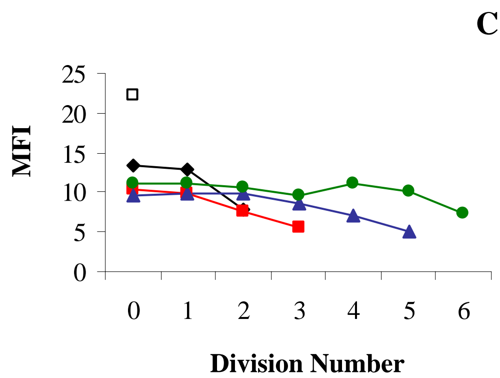
CML Patients

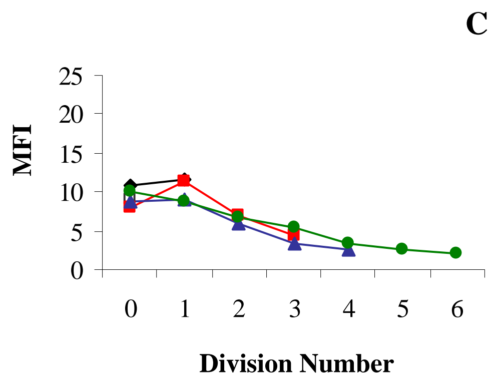












culture. In Normal A, a decrease in CD38 expression was observed with a corresponding increase in cell division number, and decreased from an average MFI of 16 units in division 0 to an MFI of 4.8 units in division 6. In Normals B and C, the level of CD38 expression was relatively unchanged by culture duration or cell division over the 4 days of culture (Figure 3.13). This excludes day 1 of culture in Normal B and day 0 of culture in Normal C, where expression was higher in division 0 relative to other days of culture (Figure 3.13).

CML patients also displayed similar profiles of CD38 expression over the culture period (Figure 3.13). In CML A and B, the level of CD38 expression was relatively unchanged with culture duration or cell division. The level of CD38 expression in CML C was unchanged by culture duration but decreased with cell division from an average MFI of 10 units in division 1, to 3 units in division 6 (Figure 3.13).

Similar levels of CD38 expression (MFI=10) on undivided cells were observed in CML patients and Normals B and C over the 4 days of culture (Figure 3.13). Although the level of CD38 expression by undivided cells was slightly higher in Normal A (MFI range = 14-22 units), the MFI of cells in division 6 was similar for all cultures (MFI range =3-7 units).

3.2.3.2 Changes in the Level of HLA-DR Expression

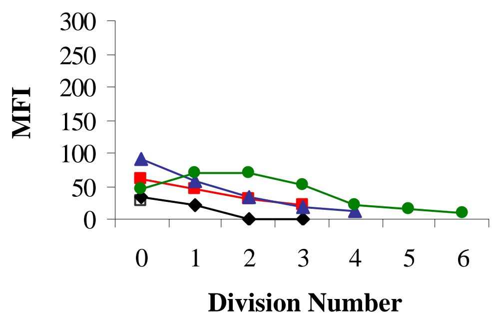
The level of HLA-DR expressed by cells from Normals A and B was unaffected by culture duration but decreased with cell division from an MFI of 55$\pm$9 units in undivided cells to an MFI of 10 units in division 6 (Figure 3.14). A different profile was seen in Normal D where an increase in HLA-DR expression that was associated with culture duration was observed in undivided cells. For example, in Normal D, the MFI of undivided cells at the initiation of the culture was 5 units and on day 4 of culture measured 240 units. Despite these variations, similar levels of HLA-DR were observed in all normal donors by division 4, with an average MFI of 10$\pm$0.1 units (Figure 3.14).

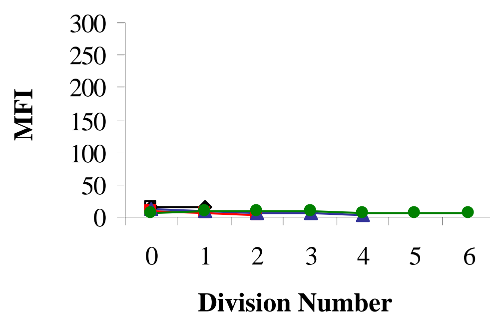
Similar profiles of HLA-DR expression were observed for all CML patients, with freshly isolated cells expressing HLA-DR at an MFI value of 18$\pm$2 units (Figure 3.14). This low level of expression was observed over the duration of the culture and was not affected by

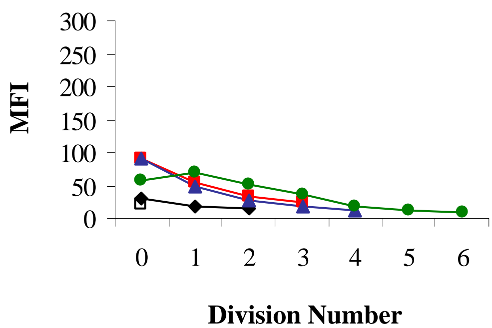
Figure 3.14 Analysis of HLA-DR Expression on Normal and CML $CD34^+$ Cells. $CD34^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for HLA-DR expression using flow cytometry. The mean fluorescence intensity (MFI) of cells that were positive for HLA-DR expression in each cell division was analysed, and plotted over 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 3 normal donors and 3 CML patients are displayed. The level of HLA-DR expression decreased with cell division in all normal donors, and was unaffected by culture duration in normal donors A and B. In normal donor D, an increase in HLA-DR expression by undivided cells was observed with an increase in culture duration, such that HLA-DR expression was 5-fold higher on day 4 of culture compared to normal donors A and B. A low level of HLA-DR expression was observed on cells from CML patients, and was unaffected by either culture duration or cell division.

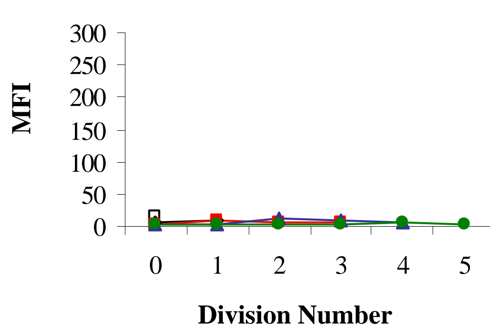
Normal Donors

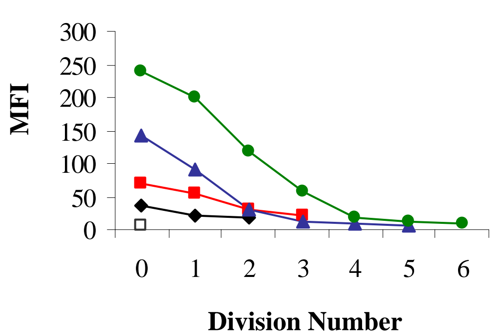
CML Patients

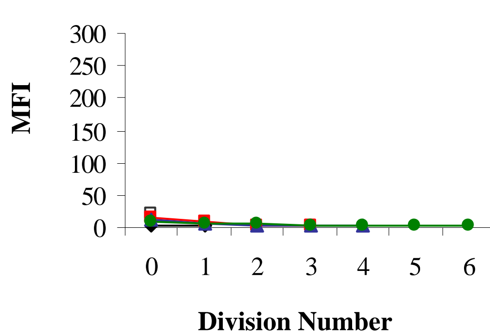












cell division (Figure 3.14).

The level of HLA-DR expression on freshly isolated cells was similar in normal donors and CML patients (Figure 3.14). Profound differences were observed over the 4 day culture period, however, and this related to a failure of undivided cell from CML patients to upregulate HLA-DR expression over the culture period (Figure 3.14). Due to a decrease in HLA-DR expression with cell division in normal donors, by division 6 the MFI of HLA-DR expression by cells from CML patients was 4.4±1.0 units versus 10.1±0.1 units in normal donors (Figure 3.14).

3.2.3.3 Changes in the Level of CD33 Expression

Variation in the profile of CD33 expression was observed between normal donors (Figure 3.15). In Normal A, the level of CD33 expressed by cells was unaffected by culture duration but increased with cell division. For example, the MFI increased from an average of 6 units in division 0 to 14 units in division 3, and expression was thereafter maintained at this level (Figure 3.15). A similar level of CD33 expression was observed on freshly isolated cells from Normal C (MFI=9 units) as Normal A (MFI=5 units) (Figure 3.15). In contrast to Normal A, CD33 expression by undivided cells in Normal C increased markedly with culture duration to a maximum MFI value of 27 units on day 4 of culture. A decrease in CD33 expression also occurred with an increase in cell division until division number 3 in Normal C, where the MFI stabilised at approximately 10 units (Figure 3.15). This plateau in CD33 expression in Normal C occurred at a similar MFI value and cell division number as observed in Normal A.

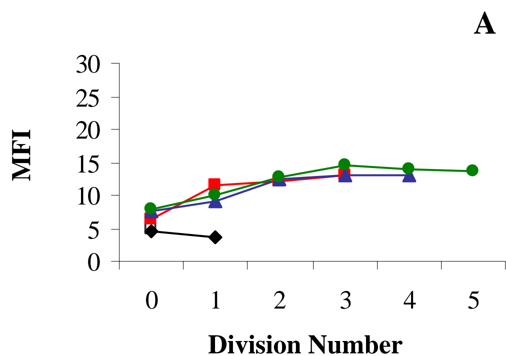
Freshly isolated cells from CML patients expressed low levels of CD33 (MFI range of 2.5-14.1 units), and variation in the effect of culture duration and cell division between patients was observed (Figure 3.15). In CML A, the MFI for CD33 expression was maintained at an average value of 5 units and was unaffected by either cell division or culture duration. In CML B, freshly isolated cells had an MFI value of 14.1 units and this increased with culture duration to an MFI of 25.4 units on undivided cells on day 4 of culture. In addition, the level of CD33 expression decreased with cell division such that by division 5, the MFI of cells was 4.8 units. In CML C, the level of CD33 expression was unchanged

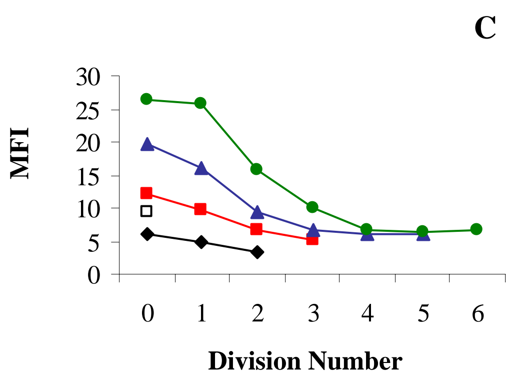
Figure 3.15 Analysis of CD33 Expression on Normal and CML CD34$^+$ Cells.

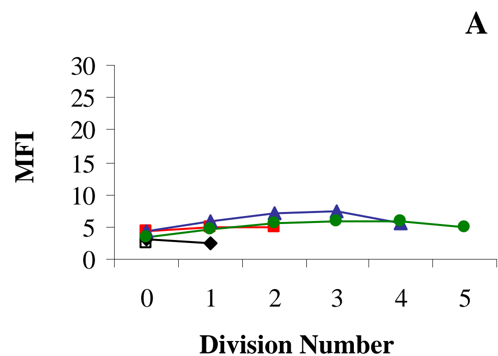
CD34$^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD33 expression using flow cytometry. The mean fluorescence intensity (MFI) of cells that were positive for CD33 expression in each cell division was analysed, and plotted over 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 2 normal donors and 3 CML patients are displayed. Variation in the level of CD33 expression by undivided cells was observed between normal donors and between CML patients. In normal donor A and CML patient C, an increase in the level of CD33 expression was observed with an increase in cell division, but was unaffected by culture duration. In contrast, the level of CD33 expression by cells from normal donor C and CML patient B increased with culture duration but decreased with cell division. The level of CD33 expression by cells from CML A was unaffected by either cell division or culture duration.

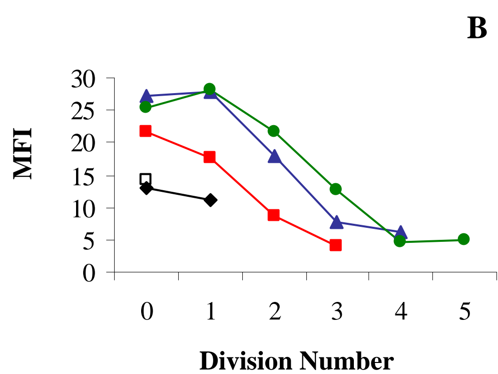
Normal Donors

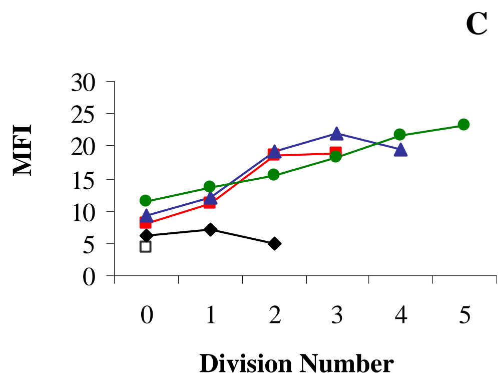
CML Patients











by culture duration but increased with cell division, similar to the profile observed in Normal A (Figure 3.15).

Variation in CD33 expression profiles was observed in both CML patients and normal donors (Figure 3.15). This variation was most apparent in divisions 0-2, and by division 3, the level of CD33 expression was similar in normal donors and CMLs A and B (MFI of 7.5 ±2.1 units). In CML C, the level of CD33 expression was approximately 200% higher in division 5 compared to other CML patients and normal donors.

3.2.3.4 Changes in the Level of CD13 Expression

In Figure 3.16, differences in the level of CD13 expression by cells from normal donors were apparent. In Normal A, CD13 expression was unchanged with cell division or culture duration. A similar level of CD13 expression was observed on freshly isolated cells from Normal D as Normal A (MFI=8.6±2.6). In Normal D, however, the level of CD13 expression on undivided cells increased from an MFI of 11.3 units on day 0 to an MFI of 69.7 units on day 4, and subsequently decreased with cell division to an MFI of 28.3 units in division 6 (Figure 3.16).

Variability in the level of CD13 expression was also observed between CML patients (Figure 3.16). In CML A, CD13 expression was maintained at an average MFI of 6.3 units over the duration of the culture. In CMLs B and C, an increase in CD13 expression on undivided cells was seen with an increase in culture duration. For example, in CML B the MFI of undivided cells increased from an MFI of 3.2 units on day 0 to an MFI of 65.3 units on day 4. In CML C the MFI of undivided cells increased from an MFI of 6.6 units on day 0 to an MFI of 103.2 units on day 4. A decrease in the level of CD33 expression with cell division in CMLs B and C was also observed, and by division 5 the MFI was 3.7 units and 14.9 units respectively (Figure 3.16).

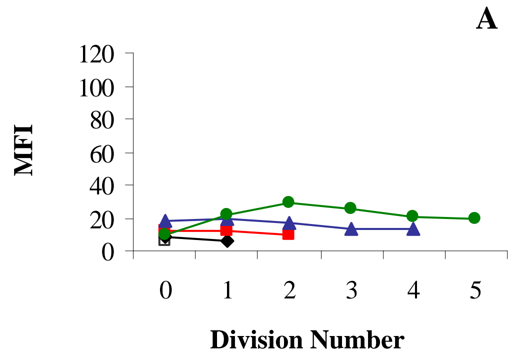
A comparison of the profiles for CD13 expression in normal donors and CML patients demonstrated variations within each group (Figure 3.16). These differences were most apparent in early division cycles and, by division 5, comparable levels of CD13 expression

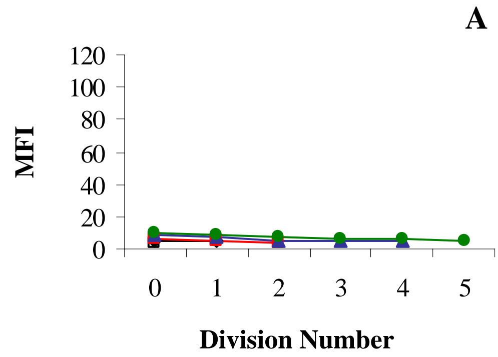
Figure 3.16 Analysis of CD13 Expression on Normal and CML CD34$^+$ Cells.

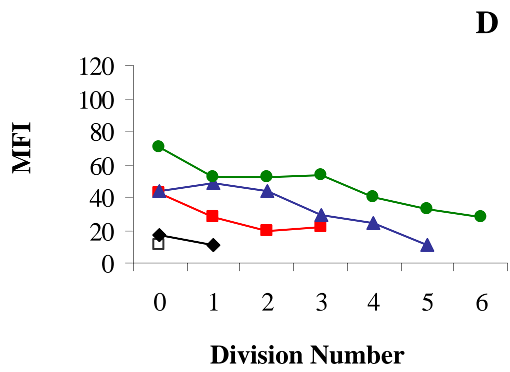
CD34$^+$ cells were isolated from the bone marrow of normal donors or peripheral blood of CML patients, stained with CFSE, and stimulated with a combination of IL-3 (10ng/mL), IL-6 (20ng/mL), G-CSF (100ng/mL), GM-CSF (100ng/mL), TPO (100ng/mL) and Flt-3 (100ng/mL) in SDM for a period of 4 days. Cells were harvested at 24 hour time points, and examined for CD13 expression using flow cytometry. The mean fluorescence intensity (MFI) of cells that were positive for CD13 expression in each cell division was analysed, and plotted over 4 days of culture (□ day 0, ◆ day 1, ■ day 2, ▲ day 3, ● day 4). Representative graphs from 2 normal donors and 3 CML patients are displayed. In normal donor A, CD13 expression was not affected by cell division or culture duration. In normal donor D, CD13 expression increased with culture duration and decreased with cell division, and expression of CD13 by undivided cells on day 4 of culture was 4-fold higher than in normal donor A. Variability in CD13 expression was also observed in CML patients. In CML patient A, expression was maintained at a low level over the duration of the culture. In CML patients B and C, CD13 expression increased with culture duration and decreased with cell division, similar to the trend observed in normal donor D.

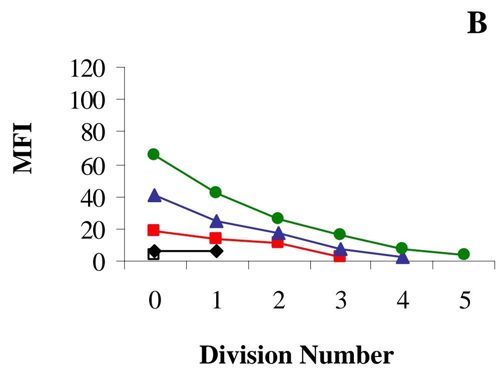
Normal Donors

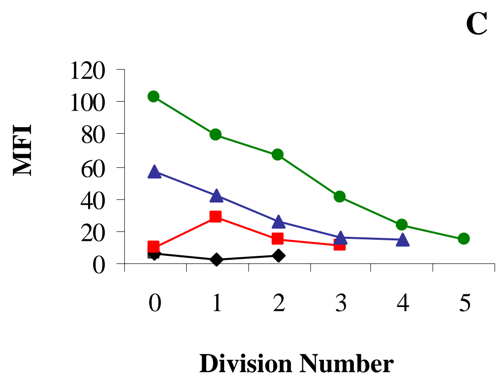
CML Patients











were observed within each group. For example, the MFI of cells in division 5 was 24.2+4.2 units in normal donors and 7.7 ± 3.5 units in CML patients (Figure 3.16).

3.3 Discussion

The effect of bcr-abl on the growth characteristics of CML progenitor cells remains controversial, and the identification of abnormalities in the proliferation and differentiation of CML cells is impeded by our limited understanding of the factors that control the behaviour of HSC under normal conditions (Lepine and Messner 1983; Bedi *et al.* 1994; Cortez *et al.* 1997; Thiele *et al.* 1997; Jonuleit *et al.* 1998). In this chapter the proliferation and differentiation of $CD34^+$ progenitor cells from normal donors was examined using CFSE analysis and compared to $CD34^+$ cells from chronic phase CML patients. Starting cells were selected on the basis of CD34 expression, as CD34 is expressed by primitive HSC, amongst other cells, and its expression is directly related to haemopoietic potential (reviewed in Pierelli *et al.* 2001). In particular, CD34 is expressed at high levels on primitive HSC while expression is downregulated during differentiation and commitment to various lineages (Pierelli *et al.* 2001).

CFSE tracking enables acquisition of detailed information on the proliferative behaviour of cells and was used in this present study to examine the effect of bcr-abl expression on the entry of cells into the cell cycle, the rate at which cells divided and the distribution of cells across each division cycle. $CD34^+$ cells from CML patients underwent equivalent rounds of division over the 4 days of culture as cells from normal donors. Variations in the percentage of starting cells from CML patients that entered cell division were apparent on day 1 of culture compared to normal controls, however, and a decrease in the proliferation index of $CD34^+$ cells from CML patients was observed on days 3 and 4 of culture. The magnitude of these deviations was variable between patients, but was of greatest magnitude in CML B.

A decrease in the proliferation index of cells from CML patients is unlikely to be a consequence of bcr-abl expression, as it has previously been reported that the proliferative potential of progenitor cells from CML patients and normal donors is similar (Bedi *et al.* 1994). Rather, the observation that the percentage of starting cells that entered the cell cycle in CML A was similar to the normal controls by division 3 suggests that cells from CML patients may have an increased threshold for stimulation and, in the absence of sufficient stimulation, the cells may remain quiescent. In CMLs B and C, cells had

impaired cell cycle entry over the 4 days of culture which may be attributable to a greater stimulation threshold in these cells, and/or an abnormality in the response of these cells to a potent cytokine stimulus. Although expression of bcr-abl has been associated with an inhibition of apoptosis (Bedi *et al.* 1994), the decreased proliferative potential of cells from CML patients observed in this chapter may also be attributable to decreased cell viability. The viability of CML cultures was lower than cultures established from normal donors over 4 days, and this is likely to be a consequence of an increased sensitivity of CML cells to cryopreservation techniques.

Studies have identified subtle differences in the response of CML cells to combinations of growth factors that include SCF, EPO and GM-CSF, suggesting that bcr-abl expression may activate signalling pathways involved in differentiation responses (reviewed in Kabarowski and Witte 2000). In this chapter, chronic phase CML cells did not exhibit an enhanced response to the growth factor combination IL-3, IL-6, G-CSF, GM-CSF, TPO and Flt-3. This growth stimulus was aimed at promoting maximal proliferation, as well as differentiation along the myeloid lineage. It would be of interest to examine the proliferation of normal and CML CD34$^+$ cells under more limited stimulation regimes, since the potent stimulus used in this study may obscure more subtle differences in the response of CML cells. For example, cellular expression of bcr-abl induces growth factor independence (Daley and Baltimore 1988). However, under maximal stimulation conditions where the majority of cells are induced to actively proliferate, enhanced proliferation due to growth factor independence would not be apparent.

In addition to comparing the proliferation of CD34$^+$ cells in CML patients and normal donors, CFSE analysis was used to examine changes in cell phenotype with cell division. CFSE has been used in the past to monitor the maturation of HSC into functional haemopoietic cells (Nordon *et al.* 1997; Pierelli *et al.* 2001). Changes in protein expression within each division cycle on each day of culture were not examined by these groups, however, such that the effect of culture duration on protein expression remained unknown (Nordon *et al.* 1997). In this chapter, CD34-selected cells were examined for changes in the percentage of cells within each cell division that expressed various molecules indicative of differentiation over 4 days of culture. This was performed to

determine whether the acquisition or loss of proteins indicative of cellular differentiation was regulated by culture duration and/or cell division under normal conditions, and whether CD34$^+$ cells from CML patients was altered in their differentiation. It was anticipated that if expression were affected by time alone, then expression of the protein of interest would be independent of the division cycle. If expression was solely regulated by cell division, then the percentage of cells expressing the protein within a given division would be expected to be identical on each of the days of culture, but vary with cell division number.

A summary of the relative influence of cell division and culture duration on the percentage of cells expressing each of the phenotypic markers from normal CD34$^+$ cell cultures is displayed in Table 3.2. Ticks denote that the expression of the particular marker was influenced by culture duration/division, while crosses indicate that the parameter had a negligible effect on expression. Data from 3 individual CML patients is also displayed, to illustrate the effect of bcr-abl expression on the regulation of haemopoietic differentiation (Table 3.2).

Table 3.2. A Summary of the Relative Contribution of Culture Duration & Cell Division Number on the Percentage of Cells Expressing Various Haemopoietic Markers.

	NORMAL CELLS		CML PATIENT A		CML PATIENT B		CML PATIENT C	
	TIME	DIVISION	TIME	DIVISION	TIME	DIVISION	TIME	DIVISION
CD34	✓	✓	✗	✓	✗	✓	✓	✓
CD38	✗	✓	✗	✓	✗	✗	✓	✓
HLA-DR^{high}	✗	✓	✓	✓	✗	✗	✗	✗
HLA-DR^{med}	✗	✓	✗	✗	✓	✓	✓	✓
CD33	✗	✓	✓	✓	✗	✓	✓	✓
CD13	✗	✓	✓	✗	✓	✓	✓	✓
CD36	✓	✓	✓	✗	✓	✓	✓	✓
CD14	✗	✓	✗	✗	✓	✓	✓	✓
CD11C	✓	✓	✓	✓	✓	✓	✓	✓
CD15	✗	✗	✗	✗	✓	✓	-	-

In contrast to results from Nordon *et al.* (1997) where it was observed that a decrease in the percentage of CD34^{+} cells occurred once cells had divided at least 3 times, a decrease in the percentage of CD34^{+} cells was observed in the absence of cell division after 1 day of culture in this chapter. These results indicate that in addition to cell division, culture duration also plays an important role in regulating the expression of CD34, as well as the expression of proteins such as CD36 and CD11c.

The results presented in this chapter demonstrate that unlike the differentiation of B cells, where isotype switching involves DNA rearrangement and deletion (Coleclough *et al.* 1980; Cory *et al.* 1980; Davis *et al.* 1980), CD34^{+} cells do not undergo a discrete program of cell division-linked differentiation where phenotype changes are unidirectional and occur in an all-or-none fashion. Variation in the expression profile of proteins such as CD33, CD36 and CD11c was also observed between normal donors further suggesting that a clearly definable program of CD34 cell differentiation does not occur. This may be attributed to heterogeneity in the stem cell pool, and supports the concept of stochastic regulation of stem cell differentiation, where the differentiation of cells is a random event (Nakahata *et al.* 1982; Novak and Stewart 1991; Mayani *et al.* 1993).

Out of the 9 phenotypic markers examined in normal donors, CD38, HLA-DR, CD33, CD13 and CD14 demonstrated a relationship between expression and cell division that was unaffected by culture duration. The percentage of cells expressing high levels of HLA-DR was found to decrease with cell division, and was associated with a corresponding increase in the percentage of cells that expressed medium levels of HLA-DR. These results suggested that cells expressing high levels of HLA-DR decreased expression to medium levels with cell division, although analysis of the MFI did not support the hypothesis that HLA-DR was lost as a consequence of division alone.

The purpose of determining whether the differentiation of CD34^{+} cells was linked to cell division and/or culture duration was to establish if a definable program of differentiation existed and whether CML CD34^{+} cells exhibited differences in this process. It was observed that the differentiation of CD34^{+} cells from CML patients was influenced by culture duration to a greater extent than their normal counterparts. For example, while the

percentage of cells from CML patients that expressed CD33, CD13 and CD14 was regulated by cell division number, as in normal donors, variation that was related to culture duration was also observed. Each CML patient was unique, and since CD34$^+$ cells from normal donors did not undergo a discrete program of differentiation that could be defined by cell division events, it was difficult to identify a specific point at which CML disrupted this differentiation program.

In most cases, differences in the percentage of cells that expressed CD38, CD14, CD33 and HLA-DR in CML patients compared to normal donors were apparent in undivided populations. This suggests that following leukaemic transformation, a dysregulation in haemopoietic differentiation commences in the early progenitor cell pool, and/or that CML CD34$^+$ cells have intrinsically aberrant expression of some markers. Alternatively, CD34$^+$ cells from CML patients may have been at a different stage of maturity at the commencement of the culture compared to CD34$^+$ cells from normal donors. This hypothesis is supported by the observation of altered expression of CD33, CD38 and HLA-DR by freshly isolated CML CD34$^+$ cells. The precise stage at which phenotypic heterogeneity in CD34$^+$ cells from CML patients is apparent may be clarified through the isolation of more primitive progenitor cells, such as CD34$^+$CD38$^-$ cell subsets. Such populations of cells were not utilised in this present study due to limitations in cell number.

Although the percentage of cells expressing various proteins in CML patients was different to normal donors at early time points and differentially regulated by cell division or culture duration, by division number 5 or 6, the percentage of cells expressing proteins such as CD33 and CD36 was often comparable to the normal controls. These results suggest that in some instances the cells appear to recover and gain appropriate expression of these markers following cell division. This is supported by the observation that myeloid cells from chronic phase CML patients are able to differentiate into mature myeloid cells which are able to perform the function of normal haemopoietic cells (Strife and Clarkson 1988).

The most profound effect of leukaemic transformation on CD34 cell differentiation was in relation to the expression of HLA-DR. In normal donors, expression of HLA-DR was regulated by cell division alone. In the 1 CML patient that did express high levels of HLA-

DR, expression was affected to a greater extent by time than cell division number. Likewise, the percentage of cells expressing medium levels of HLA-DR in CML patients was largely influenced by culture duration, although cell division also had a marked effect. Presentation of processed antigen by HLA-DR provides 1 of the 2 signals required for activation of T cells and absence of HLA-DR expression is associated with a more primitive subset of cells (Rusten *et al.* 1994). These results suggest that decreased immune competency may be a consequence in CML patients where HLA-DR expression is at inappropriate levels. Furthermore, compromised expression of HLA-DR may be associated with an inability to present putative leukaemia specific antigens, preventing the immune system from eradicating the leukaemic clone. Analysis of more differentiated progeny should be performed to determine whether this may be the case.

CD14 is another molecule that plays an important role in immune function and is recognised as a differentiation marker for monocytes (Ziegler-Heitbrock and Ulevitch 1993). It functions as a receptor for lipopolysaccharide (LPS), inducing the production of proinflammatory cytokines such as TNF-α and IL-6 and also plays a role in monocyte adhesion (Lauener *et al.* 1990; Dentener *et al.* 1993). In 2 CML patients, dysregulation in CD14 expression occurred early, as CD14 expression was acquired by undivided cells on day 4 of culture. This result suggests that these cells acquired CD14 expression when the cells were of an immature phenotype. Assuming that ligation of CD14 on these cells was able to transduce signal, the ramifications of presumably premature expression of CD14 may include inappropriate induction of inflammatory responses in CML patients.

In summary, the results presented in this chapter demonstrate that haemopoietic differentiation is complex. The expression of proteins indicative of differentiation along the myeloid lineage by normal CD34$^+$ cells was found to be affected by a combination of cell division and culture duration. This excludes the percentage of cell expressing CD38, HLA-DR, CD33, CD13 and CD14, which were regulated by cell division alone. Since a precise differentiation pathway could not be defined using cell division events, it was not possible to identify specific points at which CML disrupted this differentiation. This was further impeded by the differential effect of bcr-abl expression on CD34$^+$ cells from each CML patient. It was apparent from these studies, however, that the differentiation of

CD34$^+$ cells from CML patients was affected to a greater extent by culture duration than normal controls. Furthermore, CD34$^+$ cells from CML patients did not display increased rates of proliferation or increased entry of cells into the cell cycle, supporting the concept that expression of bcr-abl does not obligate entry of cells into the cell cycle and does not actively stimulate proliferation.

It is intriguing that significant patient-patient variability is observed clinically in CML, including differences in disease progression, severity and response to treatment, despite the fact that the disease appears to be a consequence of a single genetic lesion. Presumably differences in the proliferation and differentiation of CML CD34$^+$ cells compared to normal cells were a consequence of bcr-abl expression, as transformation of CML cells is associated with constitutively active mitogenic signalling. This includes as constitutive phosphorylation of cytoplasmic transcription factors such as STAT-3 and STAT-5 that are involved in the response of cells to IL-6 and G-CSF (Chai *et al.* 1997; de Groot *et al.* 1999; Ward *et al.* 2000). Since CFSE is distributed between the nucleus and cytoplasm, and purified nuclei therefore display an equivalent asynchronous cell division profile to intact cells (Hasbold and Hodgkin 2000), analysis of the expression of transcription factors concurrent with cell division may provide a more sensitive means by which aberrations to the differentiation of CML CD34$^+$ cells can be identified. A further approach that may assist in the identification of aberrations to the relationship between cell division and differentiation in CML CD34$^+$ cells includes simultaneous staining of cell suspensions with multiple antibodies to enable a more defined analysis of cell populations.

Chapter 4:

EFFECT OF IMATINIB ON NORMAL HAEMOPOIESIS

4.1 Introduction

CML is a HSC malignancy where the growth of haemopoietic cells is dysregulated and, during latter stages of the disease, is associated with a loss of the cells' ability to undergo complete maturation. The hallmark of CML is the Philadelphia (Ph) chromosome, which arises from a reciprocal translocation between the long arms of chromosomes 9 and 22 to generate a hybrid gene, bcr-abl (Rowley 1973; Groffen *et al.* 1984). The tyrosine kinase activity of bcr-abl is essential for its transformation of malignant cells, and logical treatment of CML therefore involves selective inhibition of bcr-abl. Imatinib is a relatively new drug used in the treatment of CML and functions as a potent ATP-competitive inhibitor of the Abl protein kinase, thereby inhibiting the kinase activity of bcr-abl and specifically inducing apoptosis in leukaemic cells (Gambacorti-Passerini *et al.* 1997). Other imatinib targets have also been identified and include the product of normal Abelson (c-abl), platelet derived growth factor receptor (PDGFR), Abl related gene (ARG) and c-kit tyrosine kinases (Buchdunger *et al.* 1996; Carroll *et al.* 1997; Buchdunger *et al.* 2000; Okuda *et al.* 2001). It is through inhibition of c-kit that imatinib has also been used in the treatment of gastrointestinal stromal tumours (GIST) (Verweij *et al.* 2003).

The efficiency of imatinib in selectively inhibiting the growth of leukemic cells has been demonstrated *in vitro* using colony assays. Assays established using the peripheral blood or bone marrow of patients with CML displayed a 92-98% decrease in the number of bcr-abl positive colonies in the presence of 1.0μM imatinib, while colony formation from normal controls was found to be unaffected up to concentrations of 10.0μM imatinib (Druker *et al.* 1996). Further research demonstrated a dose-dependent decrease in the number of granulocyte/macrophage colonies derived from normal and CML patients in the presence of escalating concentrations of imatinib, and imatinib-specific suppression of the replating potential of granulocyte/macrophage colonies in CML but not normal donor derived cells (Marley *et al.* 2000).

Clinical trials have demonstrated that imatinib can induce complete haematological and cytogenetic responses in CML patients and prevent disease progression in GIST patients, with minimal side effects (Druker *et al.* 2001; Sawyers *et al.* 2002; Verweij *et al.* 2003). The most commonly reported side effects of imatinib therapy are haematological, and

include neutropenia, thrombocytopenia, and anaemia. The myelosuppression has been attributed to the pharmacological effect of imatinib on malignant cells and a delay before normal HSC recover from suppression by the leukaemic clone (Sawyers *et al.* 2002).

Inhibitory effects of imatinib on normal haemopoiesis have not been well characterised and it remains to be addressed whether myelosuppression observed in CML patients is due to suppression of normal haemopoiesis. A suppressive effect of imatinib on normal haemopoiesis has been observed *in vitro*, where a reduction in colony formation from normal donors was observed in cells of the erythroid lineage at 0.5μM imatinib and greater (Deininger *et al.* 1997). A reduction in the growth of granulocyte/macrophage colonies was observed at 0.25μM imatinib and greater on day 7 of culture, and at 0.1μM imatinib and greater on day 14 of culture, although this inhibition only appeared to be significant at 50.0μM imatinib and 5.0μM imatinib and greater on days 7 and 14 of culture respectively (Deininger *et al.* 1997). Inhibitory effects of imatinib on normal haemopoiesis would be predicted due to inhibition of the c-kit and PDGF receptor tyrosine kinases, as SCF/c-kit signalling promotes the growth of all haemopoietic lineages when used in combination with other growth factors (reviewed in Broudy 1997), and PDGF can stimulate multilineage haemopoietic precursor cells and erythropoiesis (Dainiak *et al.* 1983; Yan *et al.* 1993; Su *et al.* 2002). The ability of imatinib to inhibit haemopoiesis in the absence of SCF and PDGF, however, has not been fully explored.

In this chapter, the effect of imatinib on the growth of haemopoietic cells from normal donors across various haemopoietic lineages was examined *in vitro*. Culture conditions were selected that would reveal effects of imatinib that are not mediated by c-kit inhibition, by using growth factor combinations with and without SCF.

4.2 Results

4.2.1 Effect of Imatinib on Normal Colony Formation

4.2.1.1 Identification of Colony Types

Normal hemopoiesis was first evaluated in semi-solid agar assays stimulated with either 4HGF (IL-3, IL-6, G-CSF, GM-CSF) or 5HGF (IL-3, IL-6, G-CSF, GM-CSF, SCF). Colony types were discriminated by differential staining using naphthol acetate esterase to identify monocyte/macrophage colonies (reddish/brown colour), chloroacetate esterase to identify neutrophil colonies (blue colour) and non-enzymatic luxol fast blue to identify eosinophil colonies (green/yellow colour) (Figure 4.1). Stimulation of cells with either 4HGF or 5HGF resulted in growth of monocyte/macrophage (Figure 4.1A), neutrophil (Figure 4.1B) and eosinophil (Figure 4.1C) colonies. Colonies composed of mixed cell types were also frequently observed, such as neutrophil/monocyte/macrophage colonies (Figure 4.1D) and eosinophil/neutrophil colonies (Figure 4.1E).

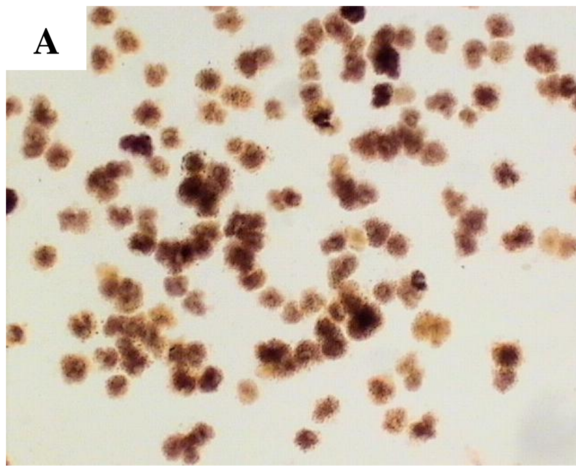
4.2.1.2 Effect of Imatinib on Total Colony Formation

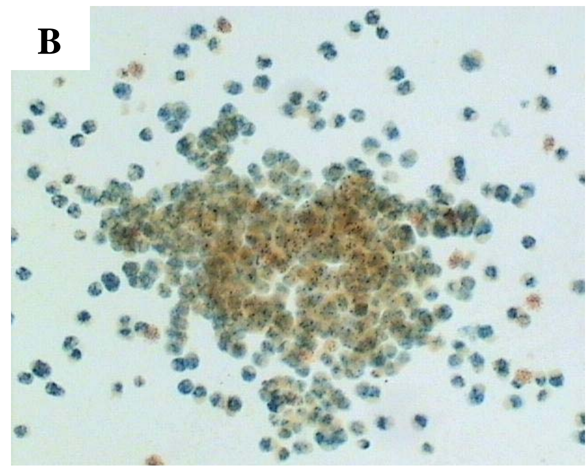
To determine whether imatinib affected colony growth in the semi-solid agar assay, cells were stimulated with 4HGF or 5HGF in the presence or absence of imatinib for a period of 14 days (Figure 4.2). A titration range of 0.3μM-50.0μM imatinib was selected for use in these initial studies, with a therapeutic dose of 400-600mg imatinib equating to an *in vitro* concentration of approximately 1.0-3.7μM imatinib (Bakhtiar *et al.* 2002; le Coutre *et al.* 2004). In the absence of imatinib, the total number of colonies that grew following stimulation with 5HGF was twice that observed when cells were stimulated with 4HGF (Figure 4.2A).

The addition of 0.3μM imatinib to cultures stimulated with 5HGF reduced colony growth to the level observed using 4HGF (Figure 4.2A). A dose-dependent relationship was seen between colony growth and imatinib concentration when either a 4HGF or 5HGF stimulus was used, and at concentrations of imatinib greater than 15.0μM, no colony growth was observed (Figure 4.2A).

Figure 4.1 Identification of Haemopoietic Colony Types using Tri-staining.

The opposing photographs are representative of the various types of haemopoietic colonies that grow on semi-solid agar following stimulation with either 4HGF (IL-3, IL-6, G-CSF, GM-CSF (each at a final concentration of 10ng/mL)) or 5HGF (IL-3, IL-6, G-CSF, GM-CSF and SCF (each at a final concentration of 10ng/mL)) for a period of 14 days. Naphthol acetate esterase staining identified monocyte/macrophage colonies (reddish brown colour) (**A**), chloroacetate esterase staining identified neutrophil colonies (blue colour) (**B**), while luxol fast blue identified eosinophil colonies (greenish-yellow colour) (**C**). Colonies containing more than one cell type were also observed, such as a combination of neutrophils and monocytes/macrophages (**D**) or a combination of eosinophils and neutrophils (**E**). A photograph was also taken at 40 times magnification to demonstrate each of the colony types in unison (**F**): neutrophil colony (**1F**), monocyte/macrophage colony (**2F**) and eosinophil colony (**3F**). Photographs A-E were taken at 100 times magnification.


A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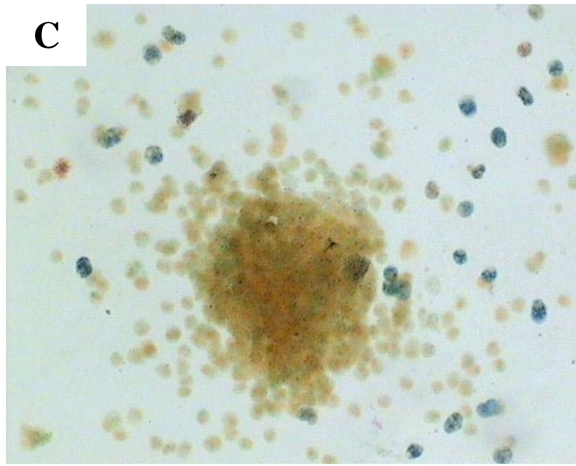
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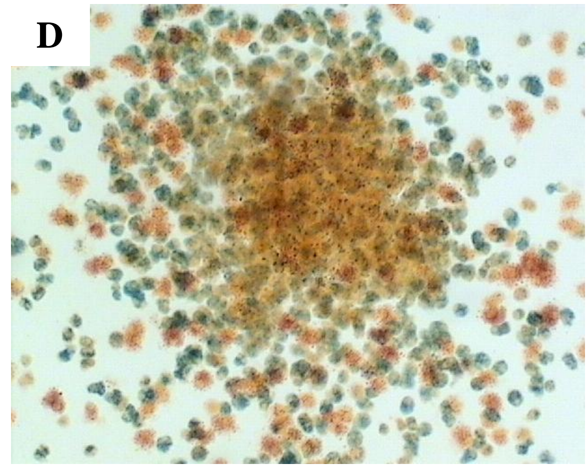
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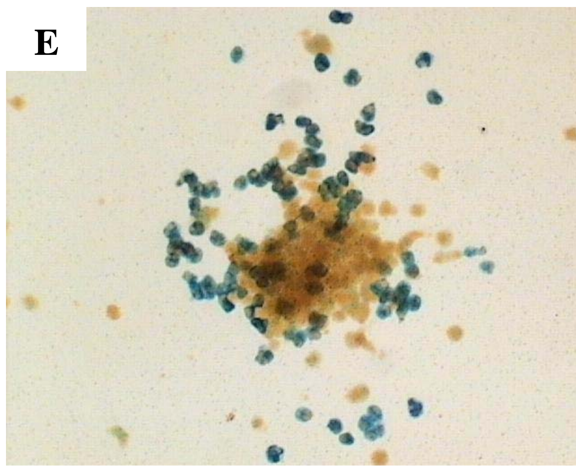
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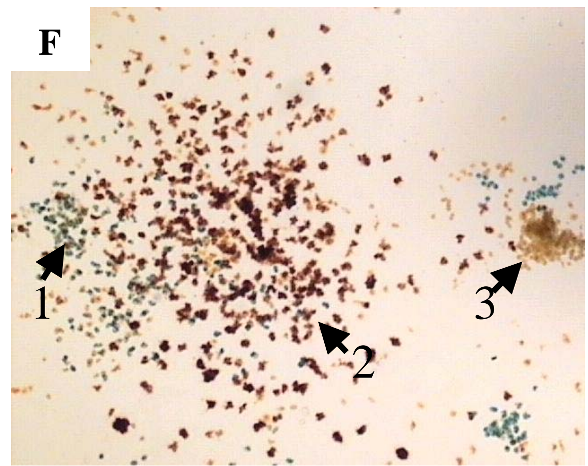
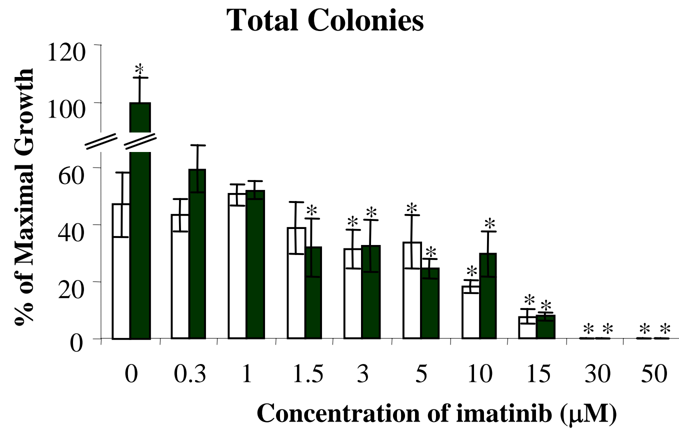
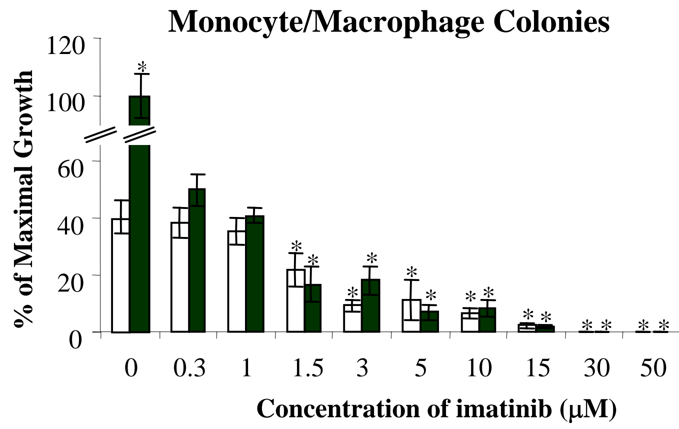

F
1
2
3

Figure 4.2 Effect of Imatinib on the Growth of Haemopoietic Colonies from Normal Bone Marrow Mononuclear Cells Following Stimulation with 4HGF or 5HGF.
Mononuclear cells were isolated from the bone marrow of normal donors and grown in semi-solid agar supplemented with either 4HGF (IL-3, IL-6, G-CSF, GM-CSF (each at a final concentration of 10ng/mL)) (□) or 5HGF (IL-3, IL-6 G-CSF, GM-CSF, SCF (each at a final concentration of 10ng/mL)) (■) for a period of 14 days. The addition of 1.5μM imatinib significantly decreased total colony number (**A**) and the number of monocyte/macrophage colonies (**B**) following stimulation of cultures with either 5HGF or 4HGF. Eosinophil colonies (**C**) were not affected until 15.0μM imatinib, and neutrophil colonies (**D**) were affected at concentrations of 3.0μM imatinib and greater. The 4HGF control was used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate. Statistical significance was determined relative to the 4HGF control (* denotes $p < 0.05$).

A



B



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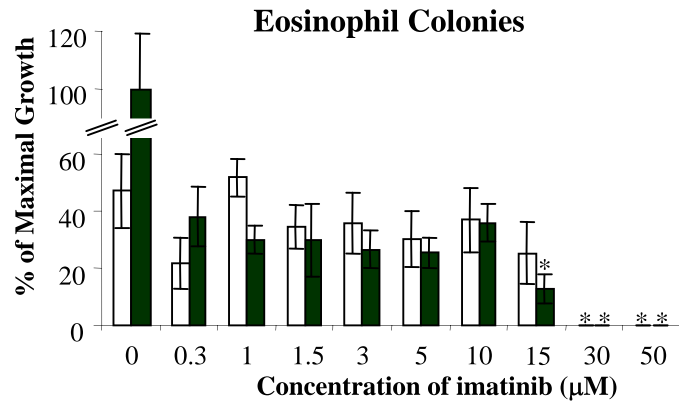
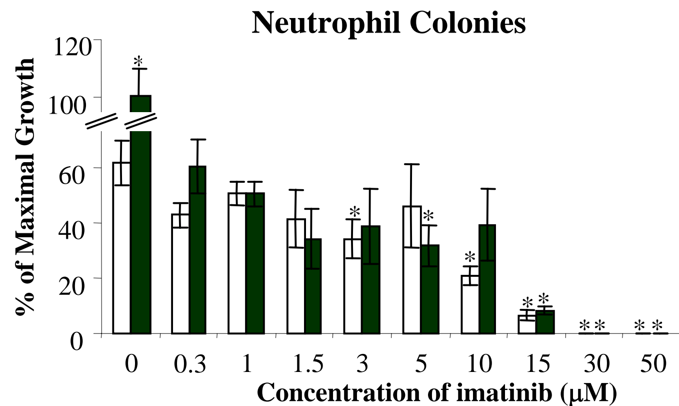


D



4.2.1.3 Effect of Imatinib on Monocyte/Macrophage Colony Formation

Naphthol acetate esterase staining was used to discriminate the effect of imatinib on monocyte/macrophage growth (Figure 4.2B). The presence of SCF in 5HGF-stimulated cultures resulted in a 150% increase in the number of monocyte/macrophage colonies compared to 4HGF cultures and this effect was abrogated by the addition of 0.3μM imatinib. A dose-dependent relationship was observed between imatinib concentration and monocyte/macrophage growth using either a 4HGF or 5HGF stimulus, and no monocyte/macrophage growth was observed at concentrations of imatinib exceeding 15.0μM (Figure 4.2B).

4.2.1.4 Effect of Imatinib on Eosinophil Colony Formation

The presence of SCF in the 5HGF stimulated cultures increased eosinophil growth by 100% relative to 4HGF cultures (Figure 4.2C). The addition of 0.3μM imatinib to 5HGF cultures reduced eosinophil growth to a level similar to that observed with 4HGF, but no further effect of imatinib on eosinophil growth was seen until the concentration of imatinib was 15.0μM or greater (Figure 4.2C).

4.2.1.5 Effect of Imatinib on Neutrophil Colony Formation

The 5HGF stimulus increased neutrophil growth by 65% relative to the 4HGF stimulus, although the addition of 0.3μM imatinib to 5HGF cultures reduced neutrophil colony formation to the level seen with 4HGF (Figure 4.2D). Further increasing the dose of imatinib had little effect on neutrophil growth until the concentration of imatinib was 15.0μM, excluding a 33% decrease in neutrophil numbers between 3.0μm and 10.0μM imatinib (Figure 4.2D).

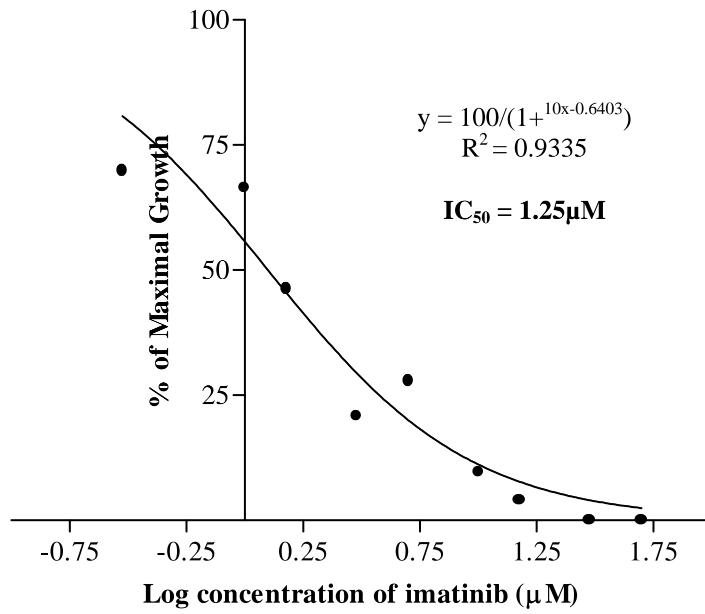
4.2.1.6 Dose Response of Colony Cultures Treated with Imatinib

The relationship between imatinib concentration and colony growth following stimulation with 4HGF was predicted according to a sigmoidal model (Figure 4.3). The equation for the line of best fit was then used to calculate the IC_{50} value for imatinib-specific inhibition of growth.

Figure 4.3 Dose Response of Normal Haemopoietic Colonies to Imatinib Treatment Following Stimulation with 4HGF.

Mononuclear cells were isolated from the bone marrow of normal donors and grown in semi-solid agar supplemented with 4HGF (IL-3, IL-6, G-CSF, GM-CSF (each at a final concentration of 10ng/mL)) for a period of 14 days in the presence or absence of imatinib. The relationship between imatinib concentration and colony growth was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of growth. The IC_{50} for monocyte/macrophage colonies was calculated to be 1.25μM imatinib (**A**), and the IC_{50} for neutrophil colonies calculated to be 6.37μM imatinib (**B**). The relationship between imatinib concentration and eosinophil colony growth could not be predicted according to a sigmoidal model. The 4HGF control was used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate.

A **Monocyte/Macrophage Colonies**



B **Neutrophil Colonies**

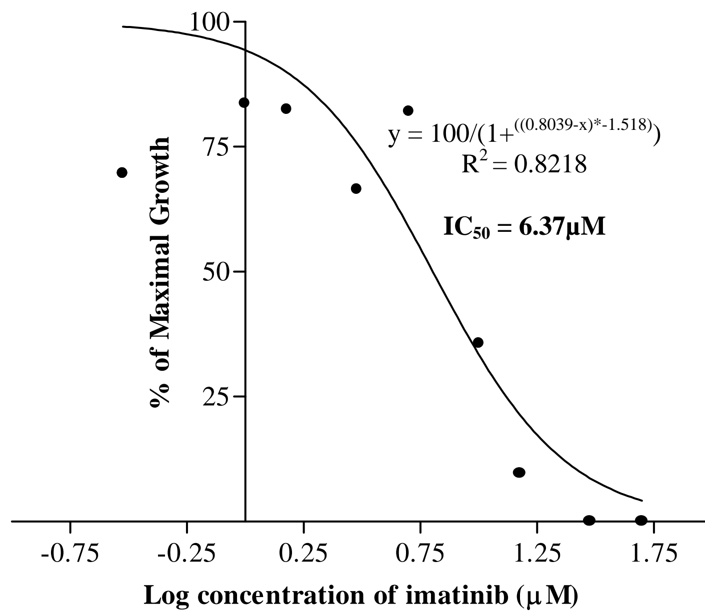


Figure 4.3A shows the imatinib dose response for monocyte/macrophage colonies, and an R^2 value of 0.93 reflected that monocyte/macrophage colony growth and imatinib concentration conformed to a sigmoidal model. From the equation for the line of best fit, the concentration of imatinib required to inhibit monocyte/macrophage growth by 50% was determined to be 1.25μM. A higher concentration of imatinib was required to inhibit neutrophil colony growth by 50%, as demonstrated by an IC_{50} value of 6.37μM imatinib (Figure 4.3B).

The relationship between logarithmic imatinib concentration and eosinophil colony growth could not be predicted according to a sigmoidal model, since eosinophil colony growth was not inhibited in a dose dependent fashion by imatinib.

4.2.1.7 Effect of Imatinib on Colony Growth Following Stimulation with a Combination of M-CSF & GM-CSF

To favour monocyte/macrophage colony growth and to determine whether the effect of imatinib on normal haemopoiesis persisted under alternative stimulation conditions, colony assays were performed using purified $CD34^+$ cells stimulated with a combination of M-CSF and GM-CSF (Figure 4.4). This approach resulted in a 350% increase in the total number of monocyte/macrophage colonies compared to colonies stimulated with 4HGF, with an average of 111 monocyte/macrophage colonies per plate versus an average of 25 monocyte/macrophage colonies per plate. The addition of 0.3μM imatinib to these cultures decreased monocyte/macrophage growth by 40% and this inhibition increased to 80% at 3.0μM imatinib (Figure 4.4A). Increasing the concentration of imatinib to 10.0μM resulted in negligible monocyte/macrophage growth.

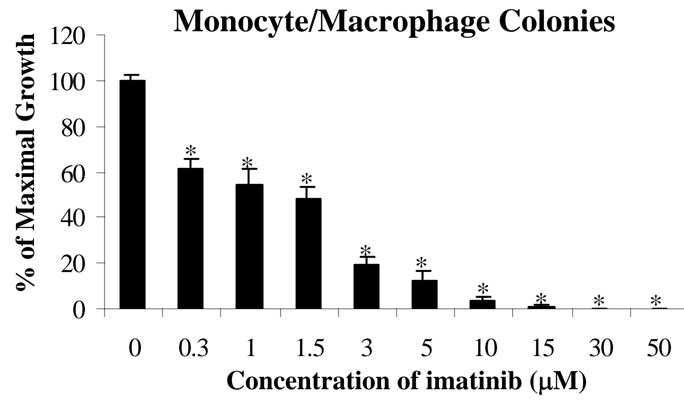
The combination of GM-CSF and M-CSF also enhanced eosinophil colony formation compared to 4HGF stimulus, with an average of 25 eosinophil colonies per plate versus an average of 10 colonies per plate. This data confirmed that eosinophil growth was unaffected by concentrations of imatinib below 10.0μM (Figure 4.4B).

The relationship between imatinib concentration and colony growth following stimulation with a combination of M-CSF and GM-CSF was predicted according to a sigmoidal model

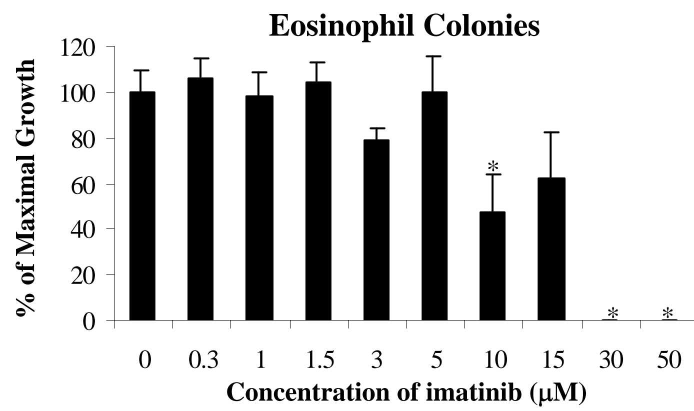
Figure 4.4 Effect of Imatinib on the Growth of Haemopoietic Colonies from Normal $CD34^{+}$ Progenitor Cells Following Stimulation with a Combination of M-CSF and GM-CSF.

$CD34^{+}$ progenitor cells were isolated from normal bone marrow and grown in semi-solid agar supplemented with a combination of M-CSF (25ng/mL) and GM-CSF (10ng/mL) for a period of 14 days. The addition of imatinib to cultures decreased the number of monocyte/macrophage colonies (**A**) at concentrations of imatinib greater than 0.3μM, while eosinophil colonies (**B**) were affected at concentration of 10.0μM imatinib or greater. Control cultures with no added imatinib were used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate. Statistical significance was determined relative to the 0μM imatinib controls (* denotes $p < 0.05$).

A



B



and was used to calculate the IC_{50} value for imatinib-specific inhibition of growth (Figure 4.5). A dose-dependent decrease in monocyte/macrophage colony formation was observed with a corresponding increase imatinib concentration and the IC_{50} value was determined to be 0.86μM imatinib (Figure 4.5A). The data conformed to a sigmoidal model, as reflected by an R^2 value of 0.92.

The concentration of imatinib required to inhibit eosinophil growth by 50% was 12.8μM (Figure 4.5B). Under these experimental conditions, eosinophil growth and imatinib concentration conformed to a sigmoidal model (R^2 = 0.91), which was in contrast to eosinophil colonies resulting from stimulation with 4HGF.

4.2.1.8 Effect of Imatinib on Colony Growth Following Stimulation with either M-CSF or GM-CSF

Therapeutic concentrations (1.0μM) of imatinib inhibited the growth of monocyte/macrophage colonies by 30-50% when GM-CSF or M-CSF alone was used to stimulate colony growth (Figure 4.6A). While the magnitude of inhibition was more marked for cultures stimulated with M-CSF, on addition of 5.0μM imatinib monocyte/macrophage growth was minimal following stimulation with either GM-CSF or M-CSF (Figure 4.6A).

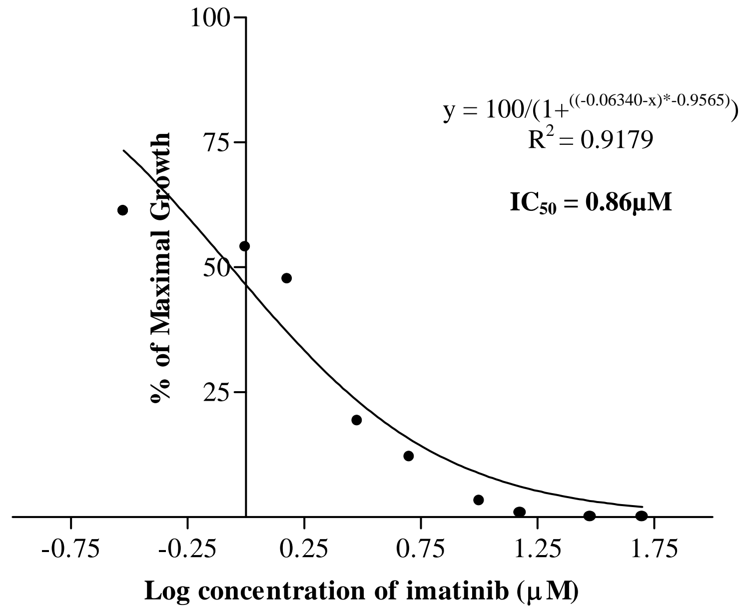
Eosinophil colony formation was observed following stimulation of cultures with GM-CSF, but not M-CSF (Figure 4.6B). The addition of imatinib to GM-CSF stimulated cultures resulted in a 20% reduction in eosinophil growth at 0.3μM and 1.0μM imatinib, but no further significant effect was observed until 5.0μM imatinib, when growth was reduced by almost 40% (Figure 4.6B). At concentrations of imatinib greater and equal to 30.0μM, no eosinophil colonies were observed. No significant neutrophil colony growth was observed in cultures stimulated with either M-CSF or GM-CSF.

A dose-dependent decrease in monocyte/macrophage growth was observed with an increase in the concentration of imatinib when cultures were stimulated with either M-CSF or GM-CSF, and was predicted using a sigmoidal model (Figure 4.7A). The inhibition was of greater magnitude in M-CSF stimulated cultures than in GM-CSF stimulated cultures,

Figure 4.5 Dose Response of Normal Monocyte/Macrophage and Eosinophil Colonies to Imatinib Treatment Following Stimulation with a Combination of M-CSF and GM-CSF.

$CD34^+$ progenitor cells were isolated from normal bone marrow and grown in semi-solid agar supplemented with a combination of M-CSF (25ng/mL) and GM-CSF (10ng/mL) for a period of 14 days. The relationship between imatinib concentration and colony growth was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of growth. The IC_{50} for monocyte/macrophage colonies was calculated to be 0.86μM imatinib (**A**), while the IC_{50} for eosinophil colonies was calculated to be 12.8μM imatinib (**B**). Control cultures with no added imatinib were used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate.

A **Monocyte/Macrophage Colonies**



B **Eosinophil Colonies**

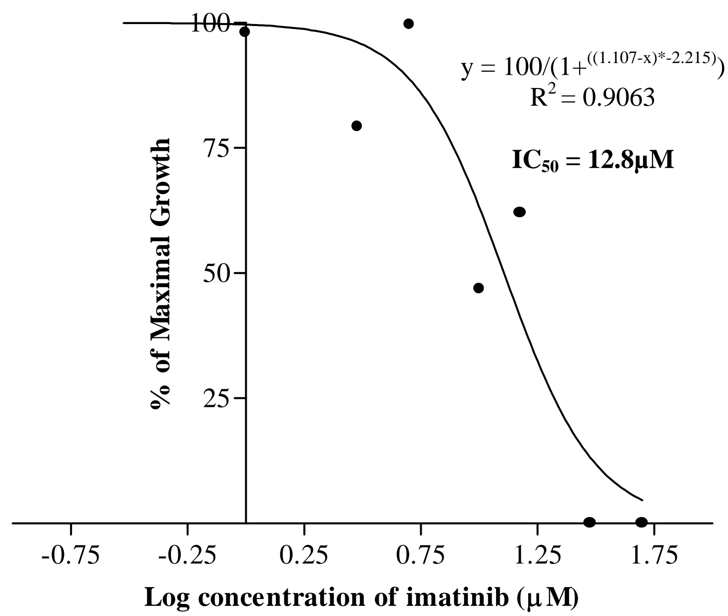
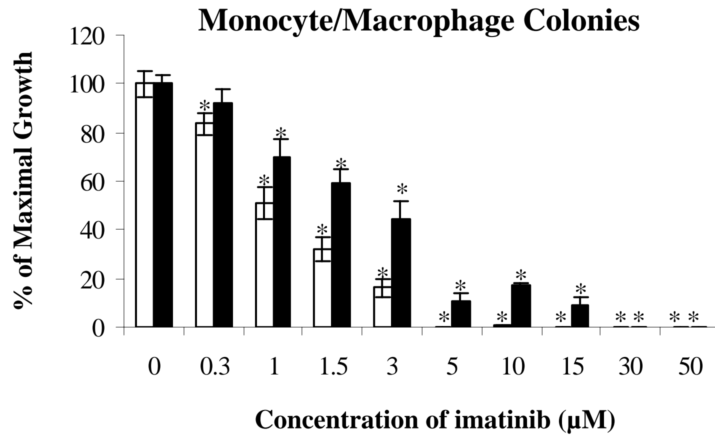


Figure 4.6 Effect of Imatinib on the Growth of Haemopoietic Colonies from Normal $CD34^+$ Progenitor Cells Following Stimulation with either M-CSF or GM-CSF.

$CD34^+$ progenitor cells were isolated from normal bone marrow and grown in semi-solid agar supplemented with either M-CSF (25ng/mL) (□) or GM-CSF (10ng/mL) (■) for a period of 14 days. The addition of imatinib to cultures significantly decreased the number of monocyte/macrophage colonies at concentrations greater than 0.3μM following M-CSF stimulation, or at concentrations greater than 1.0μM following GM-CSF stimulation (**A**). A reduction in eosinophil growth was seen at 0.3-1.0μM imatinib and this inhibition became more marked at 5.0μM imatinib or greater (**B**). No eosinophil growth was observed following stimulation of cultures with M-CSF. Control cultures with no added imatinib were used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate. Statistical significance was determined relative to 0μM imatinib controls (* denotes $p < 0.05$).

A



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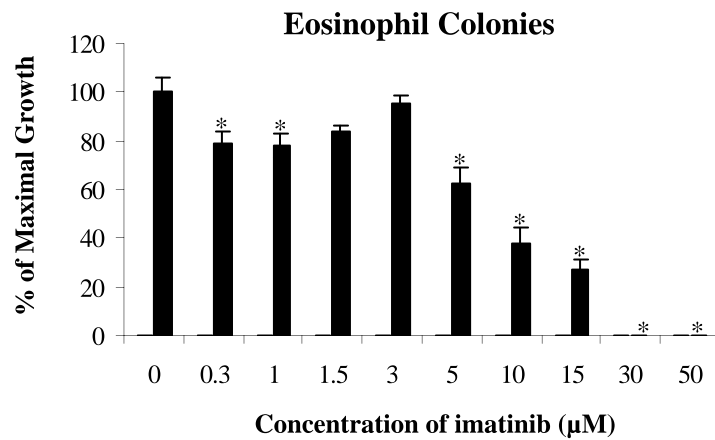
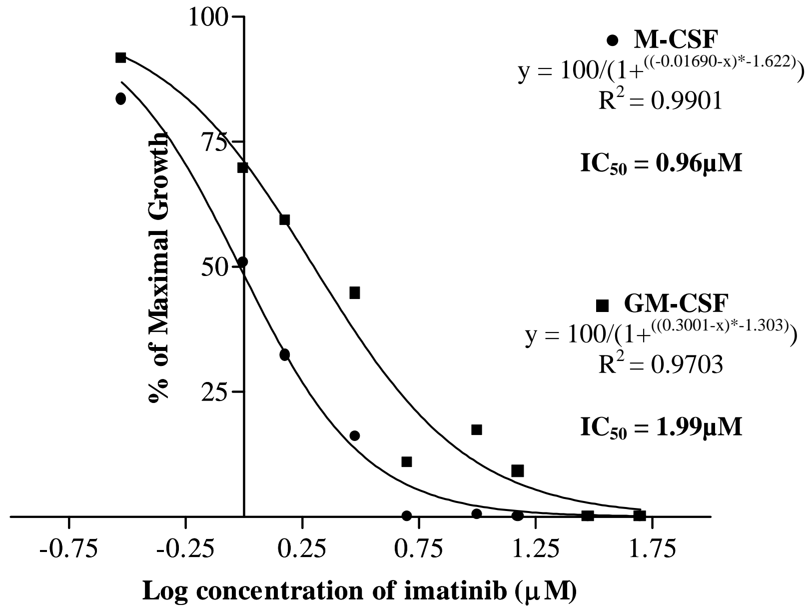


Figure 4.7 Dose Response of Monocyte/Macrophage Colonies to Imatinib Treatment Following Stimulation of Normal $CD34^+$ Cells with either M-CSF or GM-CSF.

$CD34^+$ progenitor cells were isolated from normal bone marrow and grown in semi-solid agar supplemented with either M-CSF (25ng/mL) or GM-CSF (10ng/mL) for a period of 14 days. The relationship between imatinib concentration and colony growth was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of growth. The IC_{50} for monocyte/macrophage colonies was calculated to be 0.96μM imatinib following stimulation with M-CSF (●), and 1.99μM imatinib following stimulation with GM-CSF (■) (**A**). The IC_{50} for eosinophil colonies was 7.35μM imatinib following stimulation with GM-CSF (**B**). Results represent the mean of normalised data from 2 individual experiments using different donors, and each culture condition was established in triplicate.

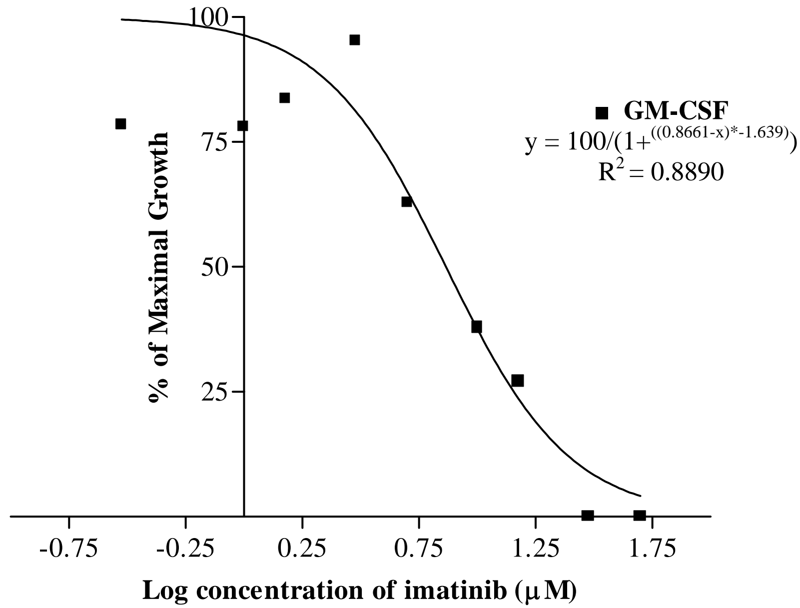
A

Monocyte/Macrophage Colonies



B

Eosinophil Colonies



and this was reflected by an IC_{50} value of 0.96μM imatinib for M-CSF cultures versus an IC_{50} value of 1.99μM imatinib for GM-CSF cultures. The IC_{50} value for eosinophil colonies from GM-CSF stimulation alone was calculated to be 7.35μM imatinib (Figure 4.7B), although an R^2 value of 0.89 demonstrated poorer conformity of the data to a sigmoidal model.

4.2.2 Effect of Imatinib on the Normal CD34⁺ Cells in Liquid Culture

To enable enumeration of cell growth concurrent with phenotype analysis, liquid cultures were established. In these experiments, $CD34^+$ progenitor cells were cultured in serum-deprived medium supplemented with 4HGF in the presence or absence of imatinib. After 3 weeks the cells were enumerated, phenotyped and assessed for viability.

4.2.2.1 Effect of Imatinib on Proliferation

Over the 3 week culture period, a 2000% increase in cell number was observed under control conditions. A dose-dependent relationship between cell growth and imatinib concentration was confirmed using this liquid culture assay, with a 25% reduction in cell number observed when 0.3μM imatinib was present for the duration of culture (Figure 4.8A). At 5.0μM imatinib the total cell number was 70% lower than control cultures and at 30.0μM imatinib cell number was below the seeded level. A reduction in cell viability was only seen at 30.0μM imatinib, where viability decreased from 80% to 60% (Figure 4.8B).

4.2.2.2 Effect of Imatinib on Cell Phenotype: Flow Cytometric Analysis

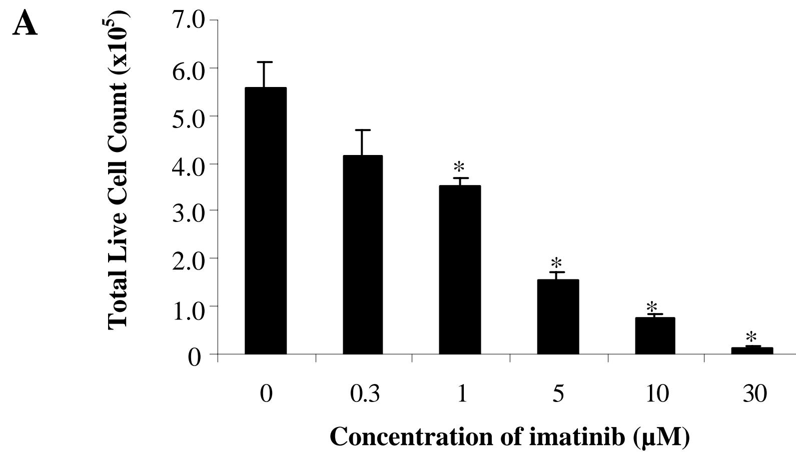
To determine whether the growth of a phenotypically distinct cell type was selected by the inclusion of imatinib in these liquid cultures, cells were examined for the expression of CD11c, CD14, CD33, CD34 and HLA-DR by flow cytometry (Figures 4.9A and 4.9B).

In the absence of imatinib, cultured cells expressed CD11c (47%), CD14 (34%), CD33 (36%) and HLA-DR (53%), and no cells retained CD34 expression (Figures 4.9A and 4.8B). The addition of 0.3μM-1.0μM imatinib to cultures reduced the percentage of cells expressing CD11c by 15%, and the percentage of cells expressing CD14 and CD33 by 25% (Figure 4.9A). HLA-DR expression was least affected by 0.3μM imatinib, with 10%

Figure 4.8 Effect of Imatinib on the Growth of Normal $CD34^+$ Progenitor Cells after Three Weeks in Liquid Culture.

$CD34^+$ cells from normal donors were stimulated with IL-3, IL-6, G-CSF and GM-CSF (each at a final concentration of 10ng/mL) for three weeks in serum deprived medium. The addition of imatinib to cultures significantly decreased total cell counts at concentrations of 1.0μM or greater (**A**). A decline in cell viability was not responsible for the decreased cell counts observed in the presence of imatinib, as a marked reduction in cell viability was only observed at the highest concentration of imatinib (**B**). Results are representative of three individual experiments, each established in duplicate, using different donors. Statistical significance was determined relative to the 0μM imatinib control (* denotes $p < 0.05$).

A



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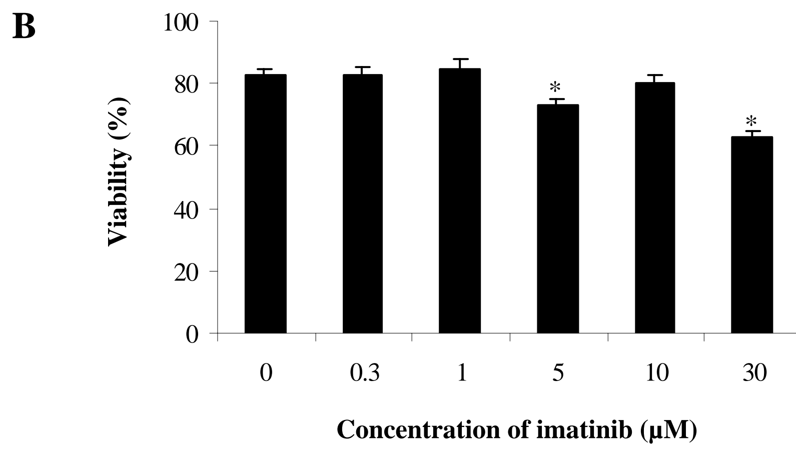


Figure 4.9A Effect of Imatinib on the Immunophenotype of Normal $CD34^+$ Progenitor Cells after Three Weeks in Liquid Culture.

$CD34^+$ cells from normal donors were stimulated with IL-3, IL-6, G-CSF and GM-CSF (each at a final concentration of 10ng/mL) in serum deprived medium for three weeks in the presence or absence of imatinib. The percentage of cells expressing CD11c, CD14 and CD33 was determined using flow cytometry, and is indicated on each histogram. A decrease in the percentage of cells expressing the phenotypic markers CD11c, CD14 and CD33 demonstrated a reduction in the percentage of more mature cells at concentrations of imatinib greater than and equal to 1.0μM. Unfilled histograms indicate background fluorescence following staining with isotype controls. The marker tool was set based on the maximum fluorescence of the isotype control. Results are representative of data from three individual experiments using different donors.

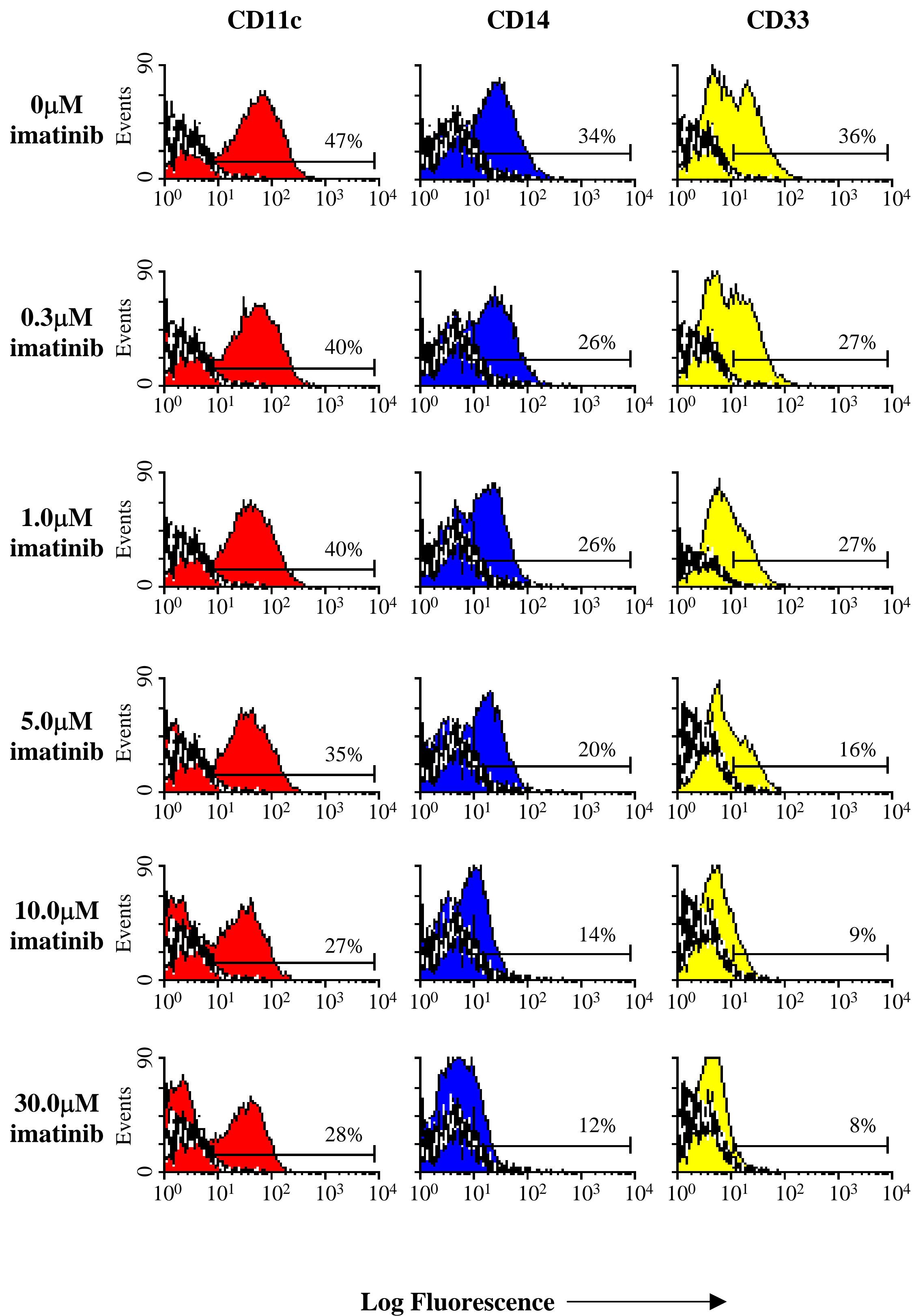
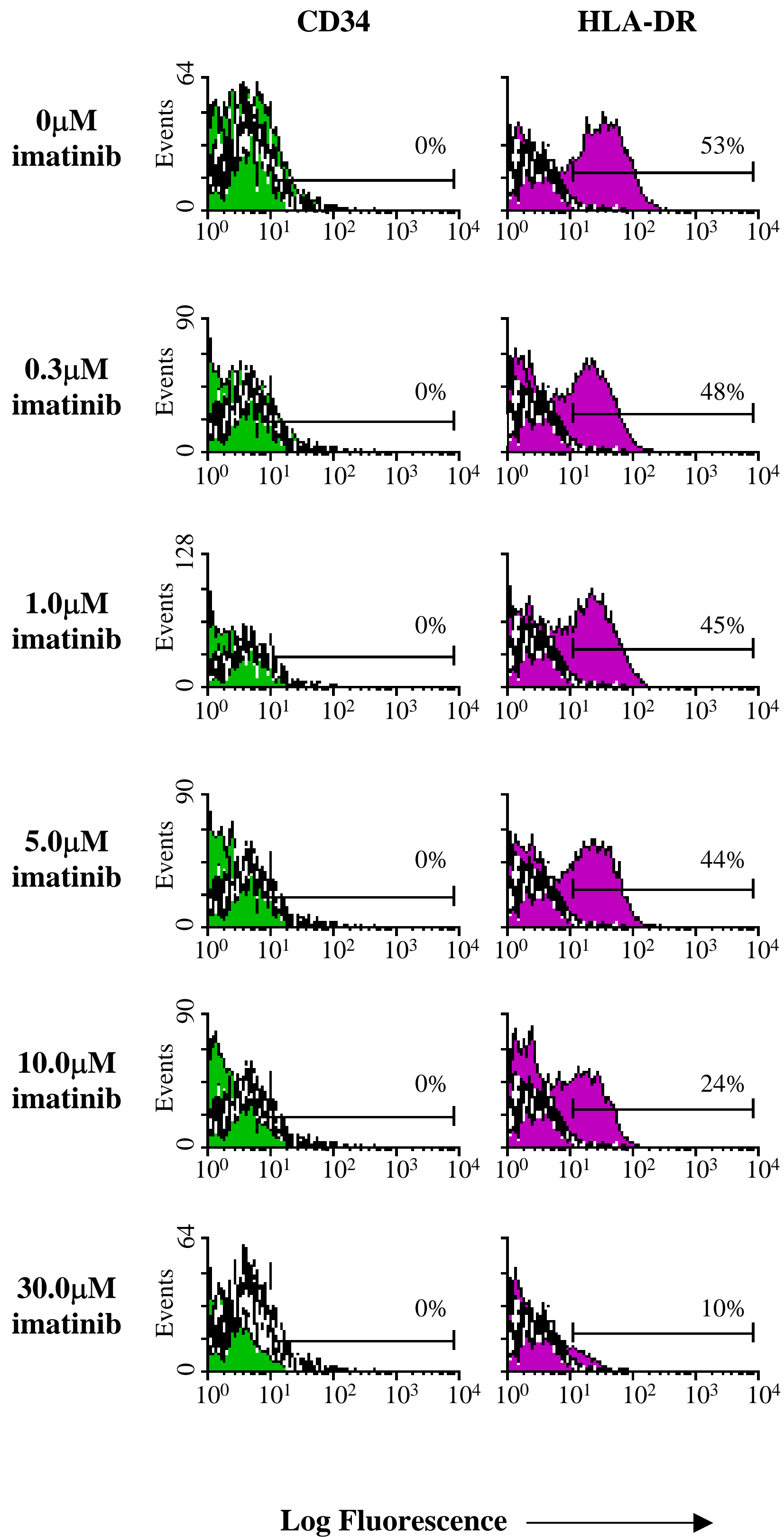

CD11c
CD14
CD33
0μM imatinib
0.3μM imatinib
1.0μM imatinib
5.0μM imatinib
10.0μM imatinib
30.0μM imatinib
Events
90
0
47%
34%
36%
40%
26%
27%
40%
26%
27%
35%
20%
16%
27%
14%
9%
28%
12%
8%
10⁰
10¹
10²
10³
10⁴
Log Fluorescence

Figure 4.9B Effect of Imatinib on the Immunophenotype of Normal CD34$^+$ Progenitor Cells after Three Weeks in Liquid Culture.

CD34$^+$ cells from normal donors were stimulated with IL-3, IL-6, G-CSF and GM-CSF (each at a final concentration of 10ng/mL) in serum deprived medium for three weeks in the presence or absence of imatinib. The percentage of cells expressing CD34 and HLA-DR was determined using flow cytometry, and is indicated on each histogram. A decrease in the percentage of cells expressing HLA-DR demonstrated a reduction in the percentage of more mature cells at concentrations of imatinib greater than and equal to 1.0μM. No cells retained CD34 expression. Unfilled histograms indicate background fluorescence following staining with isotype controls. The marker tool was set based on the maximum fluorescence of the isotype control. Results are representative of data from three individual experiments using different donors.



CD34
HLA-DR
0μM imatinib
0.3μM imatinib
1.0μM imatinib
5.0μM imatinib
10.0μM imatinib
30.0μM imatinib
Events
0%
53%
48%
45%
44%
24%
10%
Log Fluorescence

less cells expressing this marker in the presence of 0.3μM imatinib than in control cultures (Figure 4.9B).

In cultures treated with 5.0μM imatinib, the percentage of cells expressing CD11c, CD14 and CD33 was 25%, 40% and 55% lower than control values respectively (Figure 4.9A). The expression of HLA-DR, however, was equivalent to the level seen at 0.3μM and 1.0μM imatinib.

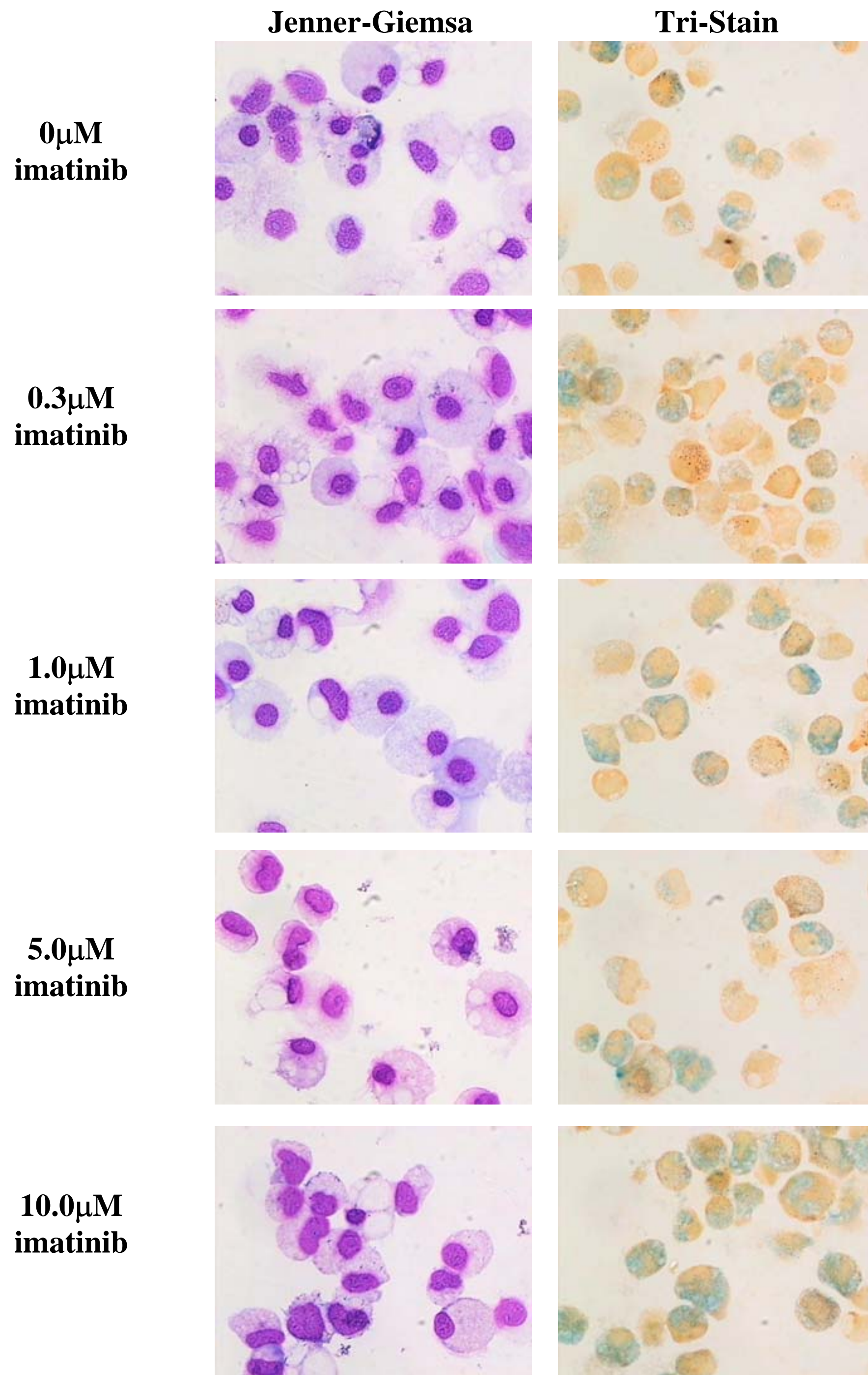
The addition of 10.0μM imatinib to cultures resulted in a further decline in the percentage of cells expressing CD11c, CD14, CD33 and HLA-DR, and this was also seen at 30.0μM imatinib. CD11c expression was observed on 43% less cells than in control cultures at 10.0μM-30.0μM imatinib, and the percentage of cells expressing HLA-DR decreased by 55% at 10.0μM imatinib (Figures 4.9A and 4.9B). CD14 and CD33 expression were most notably affected by treatment with high concentrations of imatinib, with no cells retaining expression of these markers at 10.0μM or 30.0μM imatinib (Figure 4.9A). Cells expressing CD34 were not detected in any of the cultures (Figure 4.9B).

4.2.2.3 Effect of Imatinib on Cell Phenotype: Jenner-Giemsa Staining

To further characterise the cell type(s) comprising the bulk liquid cultures, Jenner-Giemsa staining and was performed on cytospin preparations (Figure 4.10). Jenner-Giemsa staining demonstrated a cell morphology that was typical of immature myeloid cells such as promyelocytes. The absence of a significant number of cells with a morphology consistent with mature neutrophils, monocytes, macrophages and/or eosinophils from control cultures suggested that limited cell maturation had taken place (Figure 4.10).

Tri-staining of cytospin preparations was also used to examine the cellular composition of the bulk liquid cultures (Figure 4.10). In control cultures, approximately 50% of cells positively stained with naphthol acetate esterase and 50% with chloroacetate esterase, indicative of monocyte/macrophages and neutrophils respectively. No cells stained with the eosinophil-specific stain, luxol fast blue.

Figure 4.10 Effect of Imatinib on the Differentiation of Normal CD34$^+$ Cells Stimulated with IL-3, IL-6, G-CSF and GM-CSF for Three Weeks in Liquid Culture. Normal CD34$^+$ progenitor cells were grown in liquid culture in the presence of IL-3, IL-6, G-CSF and GM-CSF (each at a final concentration of 10ng/mL) for a period of three weeks. Cytospin preparations of cells were stained using Jenner-Giemsa or tri-stain (naphthol acetate esterase, chloroacetate esterase and luxol fast blue) to assess the cellular composition of cultures. Jenner-Giemsa staining revealed that cells composing each culture were of a morphology consistent with immature myeloid cells such as promyelocytes. Tri-staining also demonstrated cellular immaturity in each of the cultures, as no cells with a morphology that resembled monocytes/macrophages, neutrophils or eosinophils were observed. A higher proportion of cells staining for chloroacetate (blue) were observed in cultures treated with 10.0µM and 30.0µM imatinib.


Jenner-Giemsa
Tri-Stain
0μM
imatinib
0.3μM
imatinib
1.0μM
imatinib
5.0μM
imatinib
10.0μM
imatinib

The addition of imatinib to liquid cultures increased the intensity of blue chloroacetate esterase staining within the cytoplasm and increased the proportion of cells displaying this blue staining pattern (approximately 65% of cells at 1.0µM imatinib, 80% at 5.0µM imatinib, and 90% at 10.0µM imatinib) (Figure 4.10). This increase in the proportion of "neutrophilic" cells in the presence of an increasing dose of imatinib occurred concomitantly with a decrease in the proportion of cells staining for naphthol acetate esterase (pale brown). These results suggest that the addition of imatinib to cultures may be inducing a shift in the proportion of granulocytes relative to cells of the monocyte/macrophage lineage (Figure 4.10).

4.2.3 Effect of Imatinib on the Differentiation of Monocytes in vitro

To determine whether imatinib inhibited the differentiation of monocytes into macrophages, or acted only on the differentiation of progenitor cells, the ability of monocytes to differentiate into macrophages in the presence of imatinib was examined. Monocyte populations of 95-100% purity were isolated from the peripheral blood of normal donors using a MACS monocyte isolation kit (Miltenyi Biotech). Cultures were established in 24 well plates in serum-deprived medium supplemented with M-CSF or GM-CSF, in the presence or absence of imatinib. After 7 days of culture, the morphology and phenotype of cells was examined.

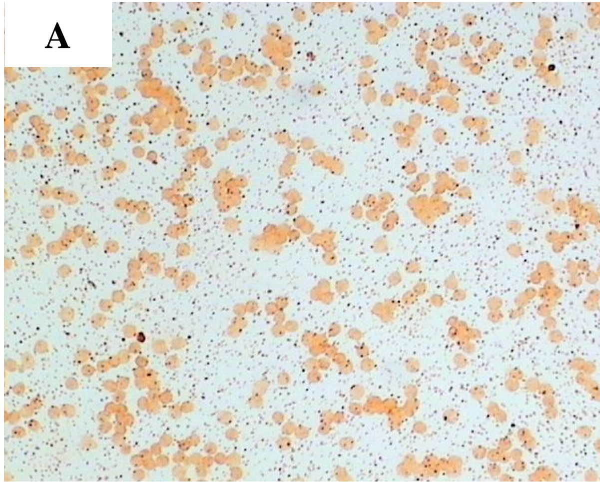
4.2.3.1 Morphological Analysis of M-CSF Stimulated Cultures

In control cultures, M-CSF stimulation of monocytes for 7 days resulted in an increase in cell size relative to freshly isolated monocytes, and spindle-like cells were also apparent due to pseudopodia formation (Figures 4.11A and 4.11B). The addition of 0.3µM imatinib to monocyte cultures stimulated with M-CSF reduced the proportion of large cells displaying this characteristic macrophage morphology (Figure 4.11C). This inhibition was even more pronounced at 5.0µM imatinib or greater (Figures 4.11E and 4.11F), where large spindle-like cells were rare and the cells were of a more rounded, monocytic morphology.

Staining of cultures with naphthol acetate esterase was performed to examine the effect of imatinib on the maturation of monocytes into macrophages. Even in the presence of

Figure 4.11 Effect of Imatinib on the Morphology of Cultured Monocytes Stimulated with M-CSF.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) for seven days. Naphthol acetate esterase staining demonstrated that imatinib decreased the differentiation of monocytes into macrophages based on morphologic properties such as the formation of pseudopodia. Monocytes (**A**) were treated with 0μM imatinib (**B**), 0.3μM imatinib (**C**), 1.0μM imatinib (**D**), 5.0μM imatinib (**E**) or 10.0μM imatinib (**F**). Photographs were taken at 100 times magnification.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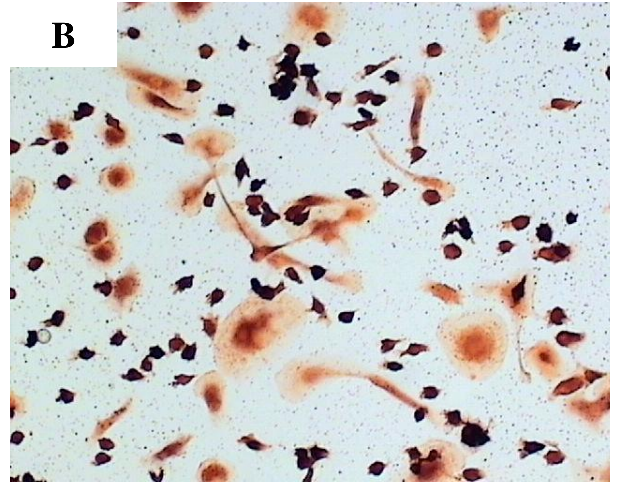
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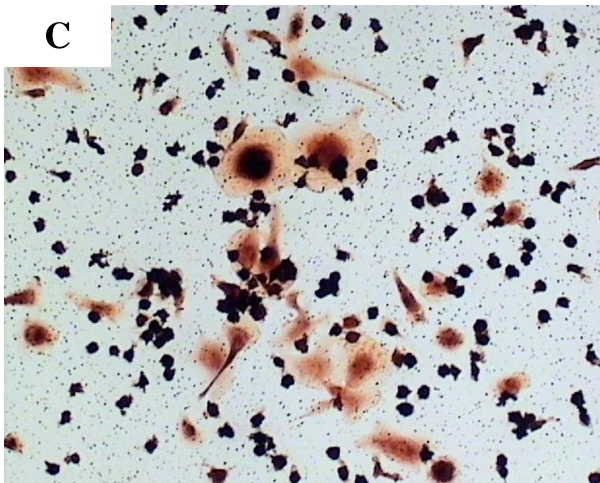
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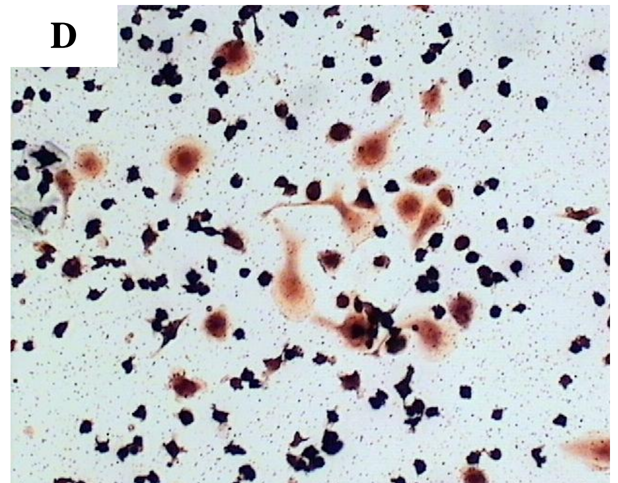
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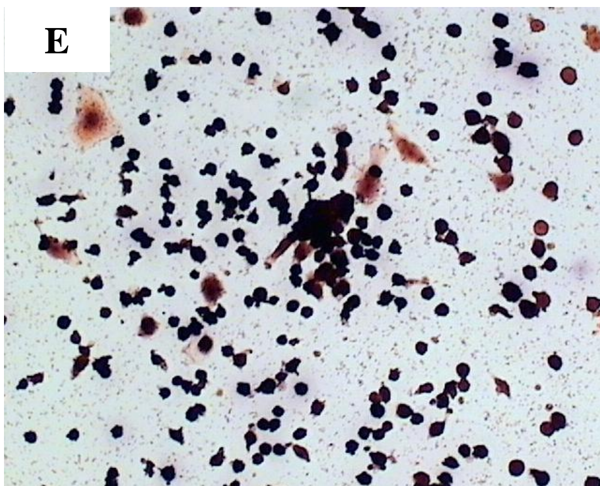
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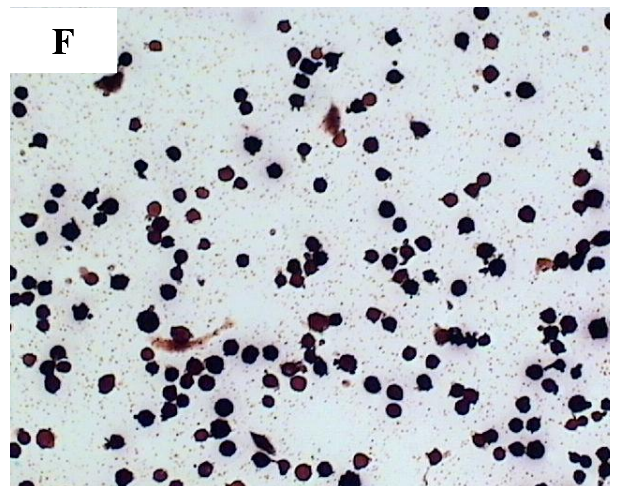

F

10.0μM imatinib, where cells lacked morphology typical of macrophages, the monocytes had undergone some degree of maturation. This was demonstrated by an increase in the intensity of naphthol acetate esterase staining relative to uncultured monocytes (Figures 4.11A and 4.11F).

4.2.3.2 Effect of Imatinib on the Immunophenotype of M-CSF Stimulated Monocyte Cultures

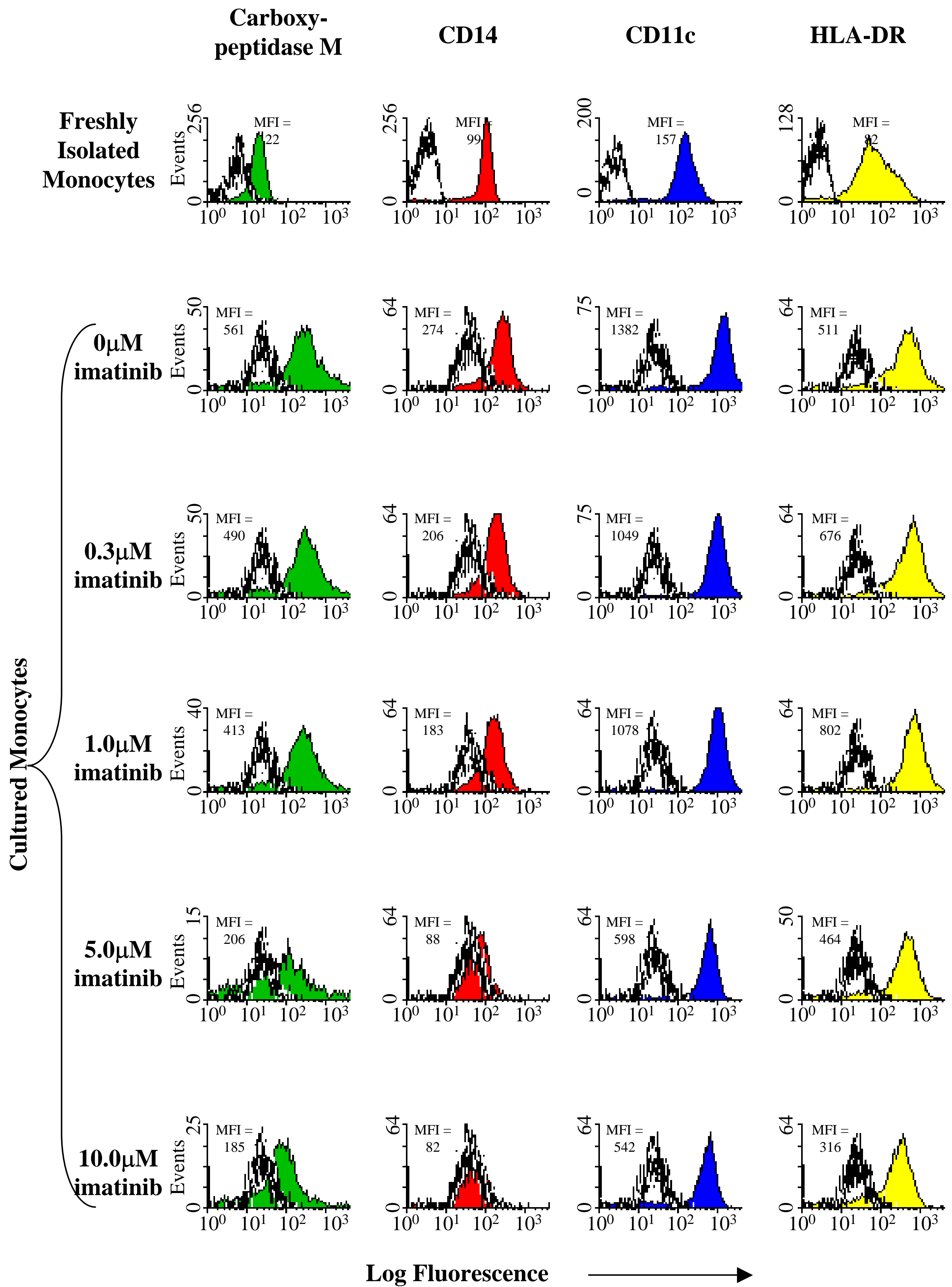
After 7 days of culture, monocytes stimulated with M-CSF in the presence or absence of imatinib were examined for the expression of proteins indicative of monocyte/macrophage maturity and function by flow cytometry, such as the expression of carboxypeptidase M, CD14, CD11c, and HLA-DR (Figure 4.12). The mean fluorescence intensities (MFI) of positive populations were then calculated to determine if imatinib affected the phenotype of cultured monocytes (CM).

The stimulation of monocytes with M-CSF for a period of 7 days increased carboxypeptidase M expression by 2450% relative to uncultured cells (Figure 4.12), with an upregulation in carboxypeptidase M expression indicative of differentiation of monocytes into macrophages (Andreesen *et al.* 1986; Rehli *et al.* 1995). The effect of imatinib on carboxypeptidase M expression by CM was also examined and expression decreased by 13% and 26% on cells cultured with 0.3μM or 1.0μM imatinib respectively (Figure 4.12). This decrease in carboxypeptidase M expression was more pronounced at 5.0μM and 10.0μM imatinib, where the MFI was 65% of control values. Even at 10.0μM imatinib, however, the level of carboxypeptidase M expression was greater than on uncultured monocytes, suggesting that these cells had undergone a degree of maturation.

CD14 expression was observed on M-CSF stimulated CM and the addition of imatinib to cultures resulted in a dose dependent decrease in CD14 expression (Figure 4.12). The MFI of CM expressing CD14 decreased by 25% at 0.3μM imatinib, and at 5.0μM imatinib the MFI was 68% of control values. CD11c expression also showed a dose response to imatinib treatment, with a 20% decrease in CD11c expression at 0.3μM and 1.0μM imatinib, and a 60% decrease at 5.0μM and 10.0μM imatinib (Figure 4.12).

Figure 4.12 Effect of Imatinib on the Phenotype of Cultured Monocytes Stimulated with M-CSF.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) for seven days. Monocytes were treated with 0μM, 0.3μM, 1.0μM, 5.0μM or 10.0μM imatinib in the presence of M-CSF stimulation, and the expression of carboxypeptidase M, CD14, CD11c and HLA-DR examined by flow cytometry. A 25-fold upregulation in carboxypeptidase M expression following 7 days of culture confirmed the transition of monocytes into macrophages under control conditions. A decrease in the expression of carboxypeptidase M, CD14, CD11c and HLA-DR demonstrated a reduction in cell maturity in the presence of imatinib. MFI represents mean fluorescence intensity. Unfilled histograms indicate background fluorescence following staining with isotype controls. Results are representative of data from three individual experiments using different donors.



Carboxy-peptidase M
CD14
CD11c
HLA-DR
Freshly Isolated Monocytes
Cultured Monocytes
0μM imatinib
0.3μM imatinib
1.0μM imatinib
5.0μM imatinib
10.0μM imatinib
Events
MFI = 22
MFI = 99
MFI = 157
MFI = 82
MFI = 561
MFI = 274
MFI = 1382
MFI = 511
MFI = 490
MFI = 206
MFI = 1049
MFI = 676
MFI = 413
MFI = 183
MFI = 1078
MFI = 802
MFI = 206
MFI = 88
MFI = 598
MFI = 464
MFI = 185
MFI = 82
MFI = 542
MFI = 316
Log Fluorescence

Treatment of M-CSF stimulated CM with imatinib increased HLA-DR expression relative to controls by 30% and 55% at 0.3µM and 1.0µM imatinib respectively (Figure 4.12). At 5.0µM imatinib HLA-DR expression was equal to controls and at 10.0µM imatinib the MFI was 40% of control values.

4.2.3.3 Morphological Analysis of GM-CSF Stimulated Cultures

The morphological differentiation of monocytes stimulated with GM-CSF was more pronounced than monocytes stimulated with M-CSF, as the majority of cells displayed an elongated, spindle-like form (Figure 4.13B). The addition of 0.3µM imatinib had little effect on the morphology of the cells (Figure 4.13C), but at 1.0µM imatinib (Figure 4.13D) there was a decrease in the proportion of spindle-like cells. This was even more apparent at 5.0µM (Figure 4.13E) and 10.0µM imatinib (Figure 4.13F), where cells with spindle morphology were virtually absent. As observed in M-CSF stimulated cultures, the intensity of naphthol acetate esterase staining was greater in GM-CSF stimulated CM than in freshly isolated monocytes irrespective of the addition of imatinib (Figure 4.13A-F), again suggesting that all cultured cells had undergone some degree of maturation.

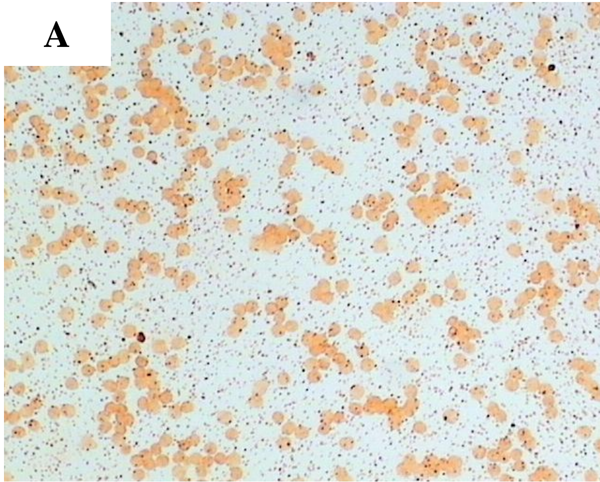
4.2.3.4 Effect of Imatinib on the Immunophenotype of GM-CSF Stimulated Monocyte Cultures

A 90% upregulation in carboxypeptidase M expression was observed on monocytes stimulated with GM-CSF for a period of 7 days (Figure 4.14). This upregulation was not as marked as that observed following stimulation of monocytes with M-CSF, and a decrease in carboxypeptidase M expression was not observed following treatment of cultures with 0.3µM or 1.0µM imatinib (Figure 4.14). At 5.0µM and 10.0µM imatinib, however, the MFI of cells expressing carboxypeptidase M was 55% of control values.

In contrast to CM stimulated with M-CSF, CM stimulated with GM-CSF displayed very low levels of CD14 expression. The addition of imatinib to cultures reduced CD14 expression such that no CD14 expression was observed at concentrations of 5.0µM imatinib and greater (Figure 4.14).

Figure 4.13 Effect of Imatinib on the Morphology of Cultured Monocytes Stimulated with GM-CSF.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) for seven days. Naphthol acetate esterase staining demonstrated that imatinib decreased the differentiation of monocytes into macrophages based on morphologic properties such as the formation of pseudopodia. Monocytes (**A**) were treated with 0μM imatinib (**B**), 0.3μM imatinib (**C**), 1.0μM imatinib (**D**), 5.0μM imatinib (**E**) or 10.0μM imatinib (**F**). Photographs were taken under at 100 times magnification.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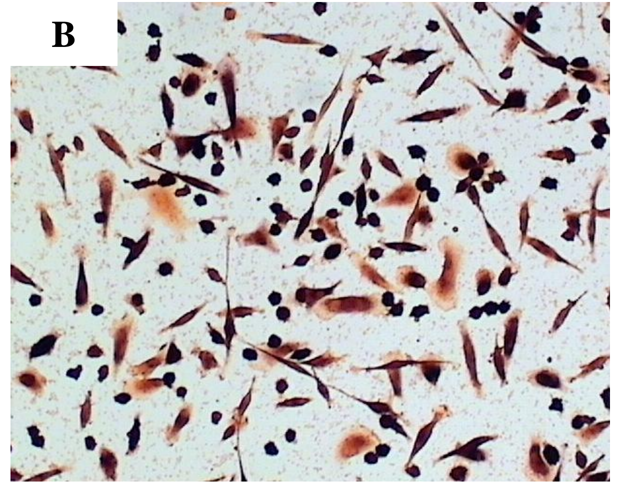
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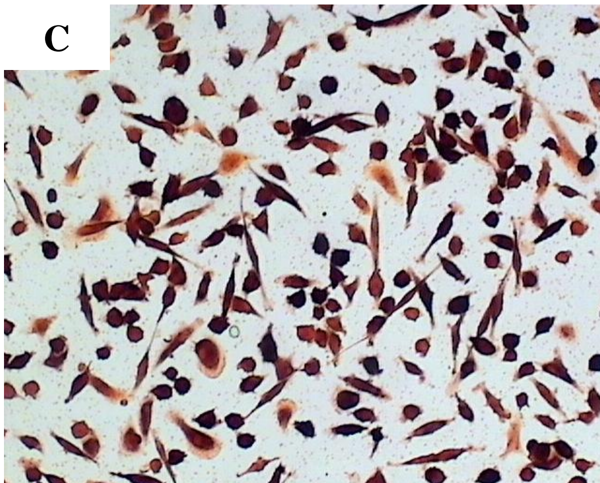
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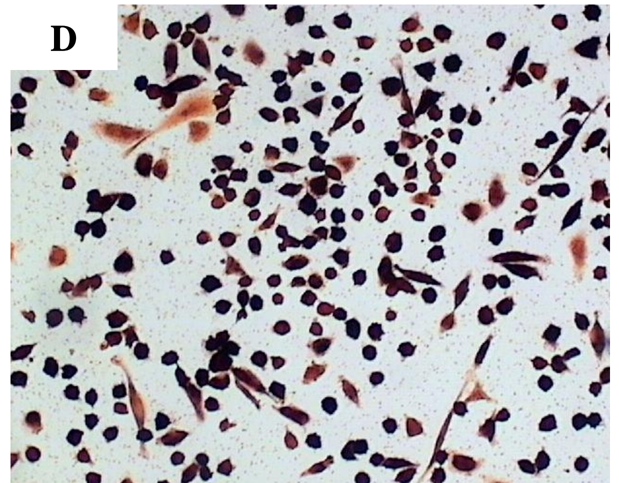
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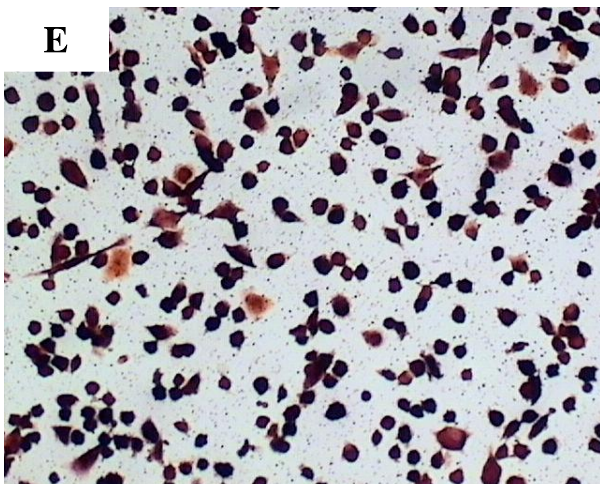
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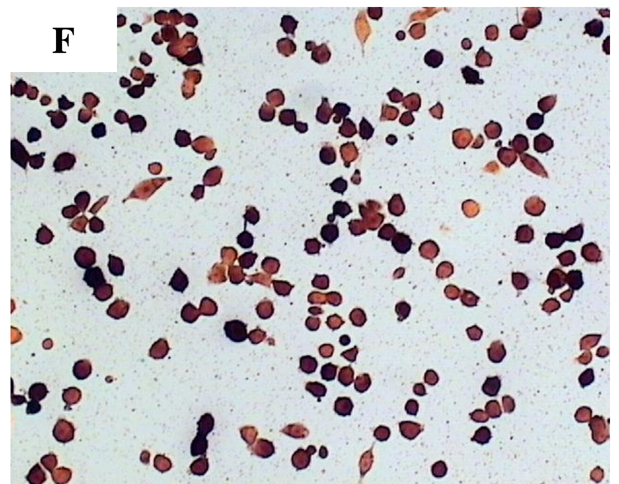
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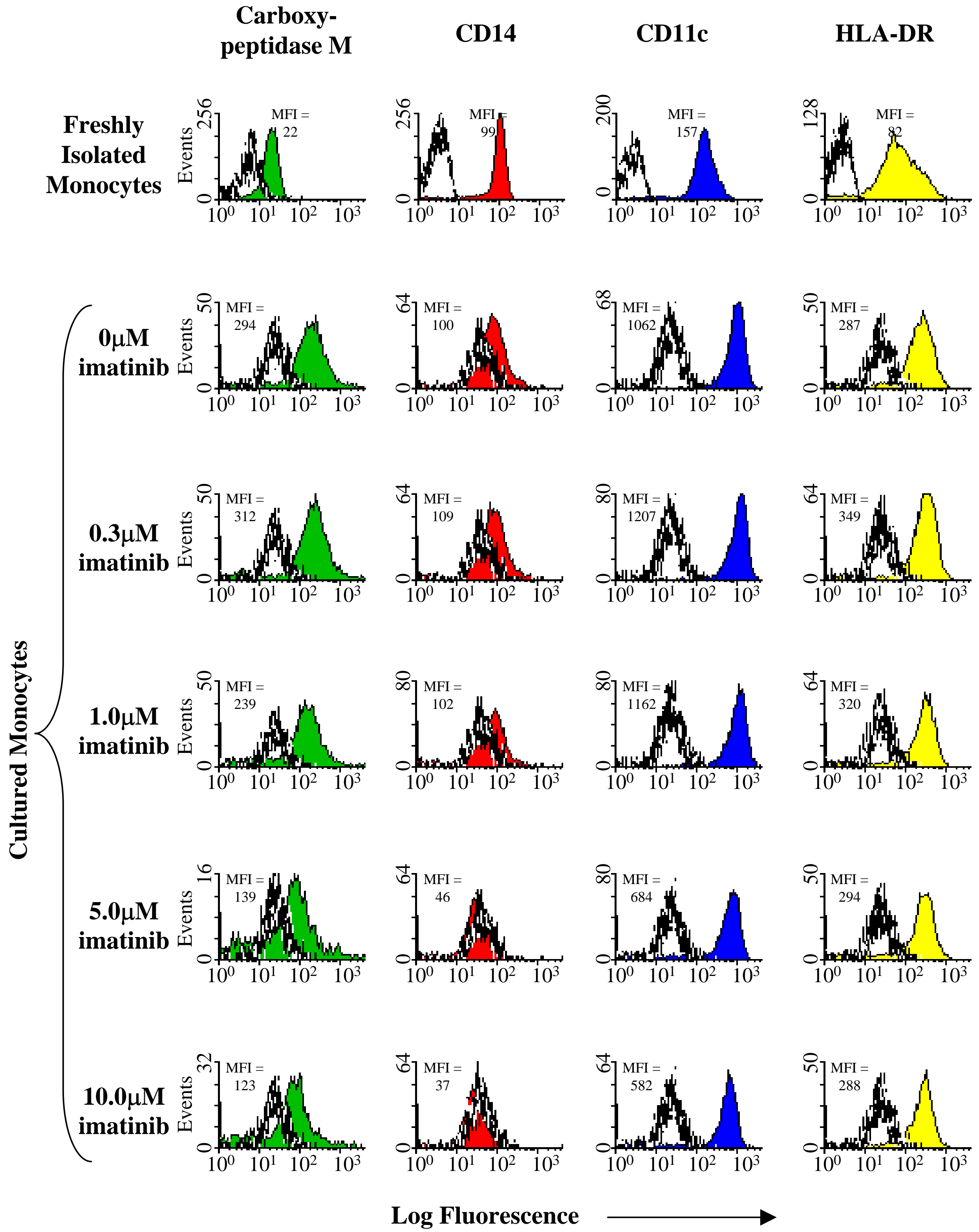
Figure 4.14 Effect of Imatinib on the Phenotype of Cultured Monocytes Stimulated with GM-CSF.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL). Monocytes were treated with 0μM, 0.3μM, 1.0μM, 5.0μM or 10.0μM imatinib for a period of seven days in the presence GM-CSF stimulation, and the expression of carboxypeptidase M, CD14, CD11c and HLA-DR examined by flow cytometry. A 13-fold upregulation in carboxypeptidase M expression following 7 days of culture confirmed the transition of monocytes into macrophages under control conditions. A decrease in the expression of carboxypeptidase M, CD14, CD11c and HLA-DR demonstrated a reduction in cell maturity in the presence of imatinib. MFI represents mean fluorescence intensity. Unfilled histograms indicate background fluorescence following staining with isotype controls. Results are representative of data from three individual experiments using different donors.


Carboxy-peptidase M
CD14
CD11c
HLA-DR
Freshly Isolated Monocytes
Cultured Monocytes
0μM imatinib
0.3μM imatinib
1.0μM imatinib
5.0μM imatinib
10.0μM imatinib
Events
MFI = 22
MFI = 99
MFI = 157
MFI = 82
MFI = 294
MFI = 100
MFI = 1062
MFI = 287
MFI = 312
MFI = 109
MFI = 1207
MFI = 349
MFI = 239
MFI = 102
MFI = 1162
MFI = 320
MFI = 139
MFI = 46
MFI = 684
MFI = 294
MFI = 123
MFI = 37
MFI = 582
MFI = 288
Log Fluorescence

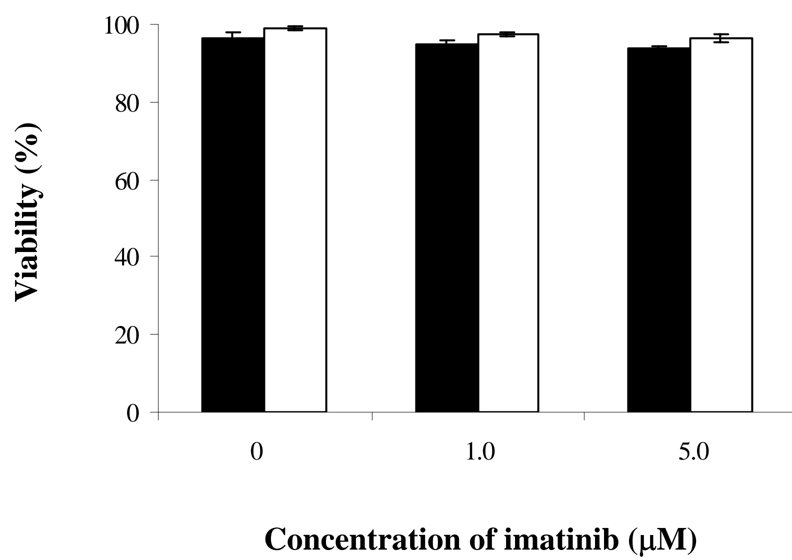
Minimal changes in CD11c expression were observed following treatment of GM-CSF stimulated CM with 0.3μM or 1.0μM imatinib. Upon addition of 5.0μM or 10.0μM imatinib, however, the MFI of CD11c positive cells was reduced by 40% relative to controls (Figure 4.14). HLA-DR expression was not affected by the treatment of GM-CSF stimulated CM with imatinib, as similar levels of expression were maintained over all concentrations of imatinib (Figure 4.14).

4.2.3.5 Effect of Imatinib on the Viability of Cultured Monocytes Stimulated with M-CSF or GM-CSF

A decrease in cell viability could not account for the effect of imatinib on the differentiation of monocytes, as viability analysis using trypan blue exclusion demonstrated that viability was consistently 95% over the range of imatinib concentrations used (Figure 4.15). This was observed for monocytes that had been stimulated with either M-CSF or GM-CSF.

Figure 4.15 Effect of Imatinib on the Viability of Cultured Monocytes Stimulated with M-CSF or GM-CSF.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) or GM-CSF (20ng/mL). Monocytes were treated with 0μM, 0.3μM, 1.0μM, 5.0μM or 10.0μM imatinib for a period of seven days in the presence M-CSF or GM-CSF stimulation, and viability examined using trypan blue. Imatinib did not affect the viability of M-CSF stimulated cultured monocytes (■) or GM-CSF stimulated cultured monocytes (□) across the concentration range tested.


100
80
60
40
20
0
Viability (%)
0
1.0
5.0
Concentration of imatinib (μM)

4.3 Discussion

Previous studies have reported that normal hemopoiesis is moderately reduced in clonogenic assays following treatment with imatinib (Deininger *et al.* 1997; Marley *et al.* 2000). Reduced colony formation was found in cells of the erythroid lineage at 0.25μM imatinib, and granulocyte/macrophage colonies at 0.1μM imatinib on day 14 of culture, although this latter result did not appear to be significant until 5.0μM imatinib where the growth of monocyte/macrophage colonies was reduced by 60% (Deininger *et al.* 1997). *In vivo*, treatment of patients with imatinib has been associated with myelosuppression and this has been attributed to eradication of the leukaemic clone (Sawyers *et al.* 2002). In this chapter, an inhibitory effect of imatinib on normal hemopoiesis involving cells of the monocyte/macrophage lineage was demonstrated at therapeutic concentrations, suggesting that myelosuppression observed in patients may be due to suppression of normal haemopoiesis.

Semi-solid agar cultures enabled analysis of the effect of imatinib on haemopoietic development. Initial cultures were stimulated with 4HGF or 5HGF, to drive the proliferation of cells along the granulocyte/macrophage lineages (Haylock *et al.* 1992). The growth-enhancing effects of SCF were completely abrogated in the presence of 0.3μM imatinib, supporting the hypothesis that signal transduction through c-kit is inhibited by imatinib (Carroll *et al.* 1997; Buchdunger *et al.* 2000). The low level of imatinib required to completely inhibit c-kit phosphorylation was consistent with results from Buchdunger *et al*. (2000), where the treatment of MO7e cells with SCF in the presence of imatinib led to inhibition of SCF-stimulated tyrosine phosphorylation with an IC_{50} value of approximately 0.1μM. In the absence of SCF, the effect of imatinib on total colony growth was significant at levels of 3.0μM and above, suggesting that abrogation of signalling through tyrosine kinases other than c-kit may be occurring.

The effect of imatinib on the growth of specific colony types was examined using non-specific esterase staining. Imatinib reduced monocyte/macrophage growth at concentrations of 1.5μM following stimulation with 4HGF, while eosinophil growth was affected at concentrations of 15.0μM and greater and neutrophil growth at concentrations greater than 3.0μM-5.0μM imatinib. The specific effect of imatinib on

monocyte/macrophage growth was confirmed using a growth stimulus of M-CSF and/or GM-CSF that favoured monocyte/macrophage colony formation, and demonstrated growth suppression with IC_{50} values of between 0.86μM and 1.99μM imatinib.

Reduced colony formation in the presence of imatinib might be explained by cell death and/or an inhibition of proliferation. A liquid bulk culture assay was therefore established using $CD34^+$ progenitor cells to confirm the inhibition of monocyte/macrophage growth and to examine the effect of imatinib on cell viability. Even at concentrations of 10.0μM imatinib, cell viability was 80%. This suggests that inhibition of cell division occurred in the presence of imatinib, rather than an increase in cell death. The possibility that cell death occurred subsequent to cell division cannot be discounted, although total cell yield demonstrated that at least the seeded cell number was recovered from all cultures until the concentration of imatinib reached 30.0μM imatinib, and viability tests revealed consistently viable cultures.

Despite significant inhibition of $CD34^+$ cell proliferation by imatinib in the liquid culture assay, the effect of imatinib on cell maturation was less striking than that observed in semi-solid agar cultures. This was attributed to the cells in liquid culture only undergoing partial maturation, and treatment with imatinib was still associated with a decrease in the percentage of cell expressing phenotypic markers such as CD11c, CD14, CD33 and HLA-DR. Failure of the cells to undergo differentiation equivalent to the level observed in the semi-solid cultures may be due to the absence of foetal calf serum from the growth medium. It was clear from cell count and viability studies in the liquid culture assay, however, that there was a generalised suppression of myeloid growth at concentrations of imatinib as low as 0.3μM.

Neither the semi-solid culture assay, nor the liquid culture assay revealed whether there was an impediment in monocyte to macrophage differentiation in the presence of imatinib. Peripheral blood monocytes placed in culture differentiate into cells that display tissue macrophage-like characteristics and it is thought that this process parallels the process of differentiation that occurs *in vivo* (Wintergerst *et al.* 1998). GM-CSF or M-CSF were used to induce the differentiation of monocytes into macrophages, as these growth factors yield

macrophages that are morphologically, phenotypically and functionally distinct from one another (Young *et al.* 1990). CD14 expression was 175% higher on CM stimulated with M-CSF than GM-CSF, with GM-CSF stimulated monocytes expressing low levels of CD14. The influence of GM-CSF and M-CSF on CD14 expression, as well as its modulation during monocyte differentiation, appears to be dependent on multiple factors including adherence, culture duration and the growth factor stimulus (Kaplan and Gaudernack 1982; Young *et al.* 1990).

Expression of CD14 and carboxypeptidase M on M-CSF stimulated CM was significantly reduced when imatinib was included in cultures. Carboxypeptidase M expression is typically upregulated following the differentiation of monocytes into macrophages (Andreesen *et al.* 1986; Rehli *et al.* 1995). The decrease in carboxypeptidase M expression in the presence of imatinib was consistent with a decrease in monocyte differentiation based on morphologic criteria. These results indicate that monocytes stimulated in the presence of imatinib do not undergo maturation equivalent to the level observed in control cultures, and suggest that these cells are morphologically and phenotypically immature.

In summary, this chapter demonstrates that imatinib inhibits the formation of monocyte/macrophage colonies from normal bone marrow progenitor cells and abrogates the differentiation of monocytes into macrophages. While liquid culture assays did not reveal a specific inhibition of differentiation along the monocytic lineage because of limited cell maturation, there was a general suppression of cell growth that was not due to a decrease in cell viability. The inhibition of monocyte/macrophage growth and differentiation occurred in the absence of exogenous PDGF and/or SCF, suggesting that inhibition of c-kit or PDGFR by imatinib is unlikely to account for these results.

The M-CSF receptor, c-fms, is a type III receptor tyrosine kinase that is closely related to c-kit and PDGFR, and plays a vital role in monocyte/macrophage development. Inhibition of this receptor by imatinib is a likely explanation for the specific inhibition of monocyte/macrophage growth and development observed by imatinib in this chapter. Published data contradicts this hypothesis, however, and suggests that c-fms is not inhibited by imatinib up to a concentration of 10.0μM (Buchdunger *et al.* 2000).

Furthermore, while some CML patients treated with imatinib exhibit cytopenia, there does not appear to be specific suppression of the monocytic lineage. The *in vivo* effect of imatinib on cells of the monocyte/macrophage lineage is difficult to assess, however, due to the short transient time monocytes spend in the blood and the location of macrophages in the tissues.

Since monocytes/macrophages play a key role in the initiation of innate and adaptive immune responses, it will be of interest to observe whether long-term administration of imatinib to CML patients will have implications for altered or depressed immune function including increased susceptibility to bacterial infections. The ability to activate cultured monocytes following imatinib treatment and the mechanism by which imatinib inhibits the growth and differentiation of monocytes/macrophages are addressed in subsequent chapters.

Chapter 5:

EFFECT OF IMATINIB ON THE FUNCTION OF CULTURED MONOCYTES

5.1 Introduction

In the previous chapter, it was demonstrated that imatinib inhibited the development of the monocyte/macrophage lineage at clinically relevant concentrations, while eosinophil and neutrophil growth were unaffected until higher concentrations of imatinib were reached. This was demonstrated through calculation of IC_{50} values, where monocyte/macrophage growth was inhibited by 50% at 0.86μM to 1.25μM imatinib, eosinophil growth at 7.35μM to 12.8μM imatinib, and neutrophil growth at 6.37μM imatinib.

Monocytes only comprise approximately 5% of circulating leukocytes, yet they provide a vital defence against pathogens. After maturation and release from the bone marrow, monocytes circulate through the blood for several hours and, under resting conditions, exist as a pool of non-activated cells. Monocytes then enter the tissues where they differentiate into macrophages and play a crucial role in both innate and adaptive immunity. The differentiation of monocytes into macrophages is associated with marked changes in morphology, biochemical parameters and effector cell function (Mayernik *et al.* 1983), and macrophages undergo additional maturation and activation under inflammatory conditions (Kaplan and Gaudernack 1982).

The biological functions of macrophages are well documented and include migration to sites of inflammation where they encounter and engulf pathogens, the production of proinflammatory mediators and the activation of adaptive immune responses following presentation of antigen to T cells. The release of macrophage activating factors such as IFN-γ by T cells (Pace *et al.* 1983), in combination with other signals, further enhances the function of macrophages. Due to the potent action of activated macrophages, the function of these cells must be carefully controlled. Signals to downregulate macrophage activation are delivered from surrounding cells such as T cells following secretion of cytokines such as IL-10 and transforming growth factor β (TGF-β), or arise from within the macrophage itself (Bogdan and Nathan 1993; Wu *et al.* 1993; Mosser 2003).

Uptake of antigen by macrophages occurs by an endocytic event that can involve receptor-mediated uptake, pinocytosis or phagocytosis (reviewed in Aderem and Underhill 1999; Watts and Amigorena 2001). Phagocytosis occurs by an actin-dependent mechanism

where binding of antigen to receptors induces rearrangement of the actin cytoskeleton and subsequently results in internalisation of the foreign particle (Aderem and Underhill 1999). To enable discrimination between foreign antigens and self-molecules, macrophages possess restricted receptors that recognise conserved motifs on pathogens such as mannose receptors, integrins and scavenger receptors (Stahl and Ezekowitz 1998; Aderem and Underhill 1999).

Antigens are also phagocytosed by complement receptors after opsonisation with complement and by Fc receptors following opsonisation with antibodies (Ravetch 1997). Opsonisation typically increases the efficiency of the interaction between the phagocytic cell and foreign particle and, by inducing receptor-mediated phagocytosis, enhances the capacity of the phagocytic system by accelerating the kinetics of phagocytosis (Roos *et al.* 2004). In contrast to the phagocytosis of complement-opsonised antigens by complement receptors, Fc receptor mediated phagocytosis of antibody-opsonised antigens is accompanied by the production and secretion of proinflammatory mediators such as reactive oxygen species and arachidonic acid metabolites (Nitta and Suzuki 1982; Muroi *et al.* 1994). Ligand binding to the mannose receptor, such as by mannose and fucose on the surface of pathogens, also induces the production of proinflammatory cytokines such as IL-6, TNF-α, GM-CSF and IL-1β (Garner *et al.* 1994; Yamamoto *et al.* 1997).

Following uptake of antigen by macrophages, the antigens are processed and presented as peptides by MHC class II molecules to T cells, thereby activating adaptive immune responses. Macrophage mediated T cell activation is regulated by secreted compounds such as TGF-β and prostaglandin E2 that suppress T cell proliferation, as well as by cell surface molecules such as CD80 and CD86 that provide a costimulatory signal and play a crucial role in the T cell - macrophage interaction (Doherty 1995).

Monocytes/macrophages can also be activated by lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. By potently activating macrophages, LPS induces the production of many inflammatory mediators such as reactive oxygen, and cytokines such as IL-6 and TNF-α, which upregulate host immune responses (Dentener *et al.* 1993; Asakura *et al.* 1996; Amura *et al.* 1998). While the

mechanisms for activation of the signalling pathways induced by LPS stimulation are not completely understood, evidence supports the idea that proinflammatory signalling induced by LPS is mediated by CD14 and an LPS-binding protein (reviewed in Ulevitch and Tobias 1995). LPS activation of monocytes/macrophages has also been shown to occur independently of CD14 (Lynn *et al.* 1993).

It was observed in Chapter 4 that monocytes cultured in the presence of imatinib appeared to be phenotypically and morphologically immature compared to control cultures. This chapter seeks to extend these findings and address whether imatinib inhibits the function of monocytes that have been cultured in the presence of imatinib. In particular, the effect of imatinib on LPS activation of cultured monocytes (CM) is examined with respect to morphology and cytokine production, as well as antigen uptake and/or stimulation of responder cells in a mixed lymphocyte reaction. These results may have important implications with regard to immune function in patients currently receiving imatinib treatment and may provide further information on the potential utilisation of imatinib in diseases of monocyte/macrophage aetiology.

5.2 Results

5.2.1 Effect of Imatinib on the Activation of Cultured Monocytes by LPS: Morphological Assessment

The effect of imatinib on the response of CM to LPS activation was examined following stimulation of CM with M-CSF, GM-CSF, or a combination of M-CSF and GM-CSF. These 2 growth factors were selected because of their ability to support the differentiation of monocytes into macrophages, but with resulting phenotypes and morphologies that are distinctly different (Young *et al.* 1990).

5.2.1.1 M-CSF Stimulated Monocytes

The effect of imatinib on the response of CM to LPS activation was initially examined morphologically (Figure 5.1). In the absence of imatinib treatment, LPS activation induced an increase in pseudopodia formation in M-CSF stimulated CM (Figure 5.1B) compared to CM that had not been treated with LPS (Figure 5.1A). Cellular aggregation was also observed following LPS activation of M-CSF stimulated CM (Figure 5.1B).

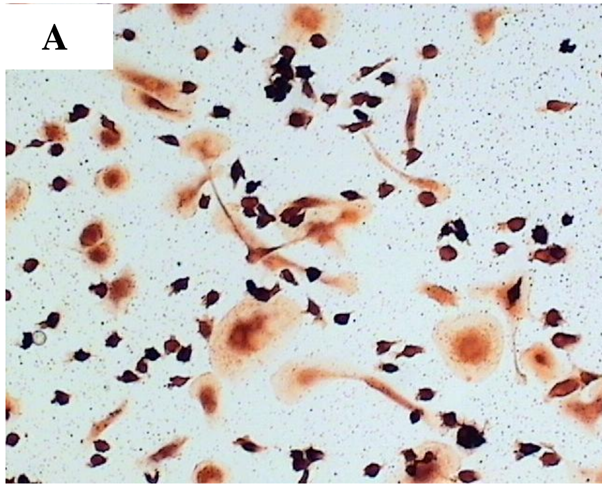
The addition of 0.3μM imatinib to LPS activated monocyte cultures decreased the length and extent of pseudopodia compared to control cultures (Figure 5.1C). At 1.0μM imatinib, cells were still spiked in morphology, although cells with long pseudopodia were absent. At 5.0μM (Figure 5.1E) and 10.0μM imatinib (Figure 5.1F) the cells were of a more rounded phenotype that is characteristic of monocytes. The cellular aggregation observed in M-CSF stimulated cultures following treatment with LPS was unaffected by imatinib treatment (Figures 5.1B-F).

5.2.1.2 GM-CSF Stimulated Monocytes

The treatment of GM-CSF stimulated monocytes with LPS for 48 hours also increased pseudopodia formation (Figure 5.2B) relative to untreated controls (Figure 5.2A). The addition of 0.3μM imatinib had little effect on LPS activation of GM-CSF stimulated CM (Figure 5.2C), however, at 1.0μM imatinib pseudopodia formation was markedly reduced (Figure 5.2D) when compared to control cultures (Figure 5.2B). At 5.0μM (Figure 5.2E) and 10.0μM (Figure 5.2F) imatinib, pseudopodia formation was limited and the cells were of a more rounded phenotype.

Figure 5.1 Effect of Imatinib on the Morphological Response of M-CSF Stimulated Monocytes to Lipopolysaccharide Activation.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) in the presence or absence of imatinib for five days. These cultured monocytes were then activated with 1μg/mL lipopolysaccharide (LPS) for 48 hours in the presence of M-CSF stimulation and imatinib treatment, and stained with naphthol acetate esterase to assess cellular morphology. In the absence of LPS stimulation and imatinib treatment (**A**), the differentiation of monocytes was typified by the formation of pseudopodia. In the presence of LPS (**B**), pseudopodia formation was enhanced and cells tended to form aggregates. The presence of 0.3μM imatinib (**C**) in LPS activated cultures reduced pseudopodia formation. At 1.0μM (**D**), 5.0μM (**E**) and 10.0μM (**F**) imatinib, cells continued to aggregate in response to LPS activation, however this occurred in the absence of pseudopodia formation. Photographs were taken at 100 times magnification.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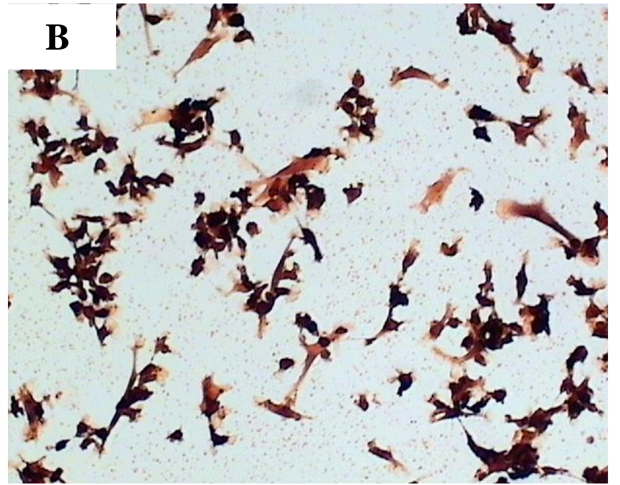
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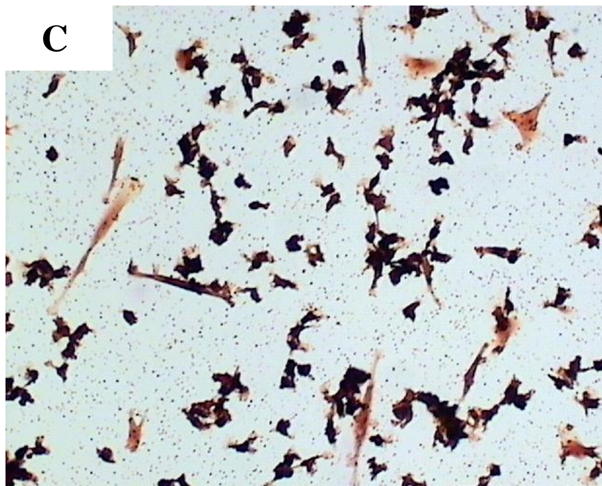
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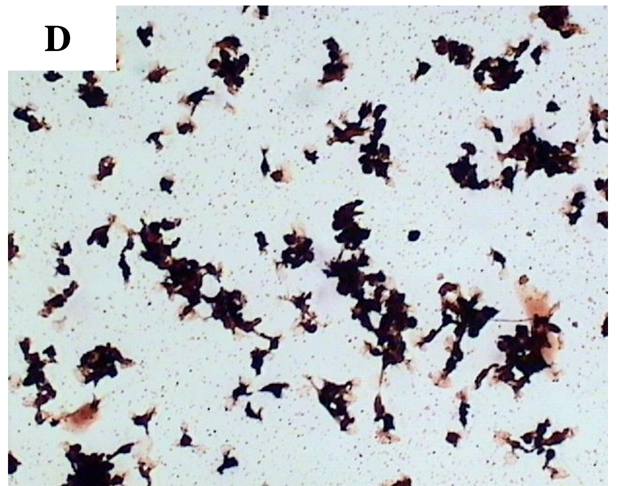
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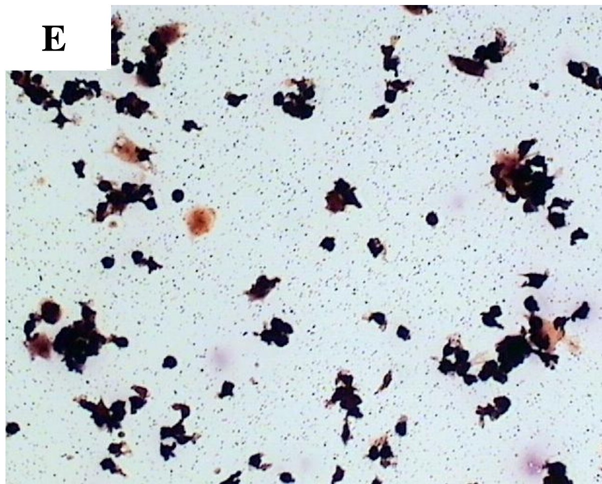
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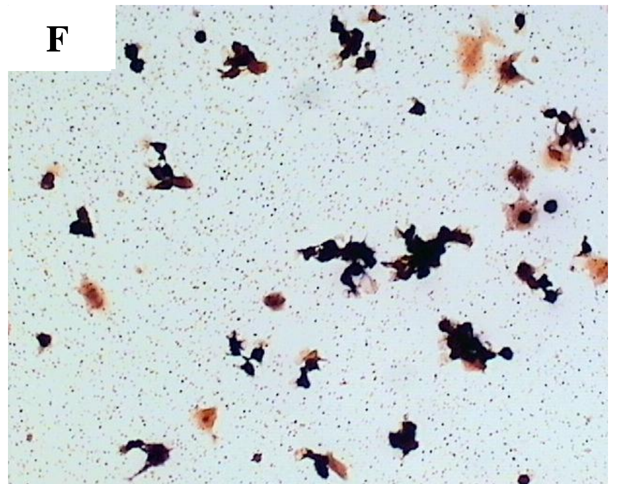
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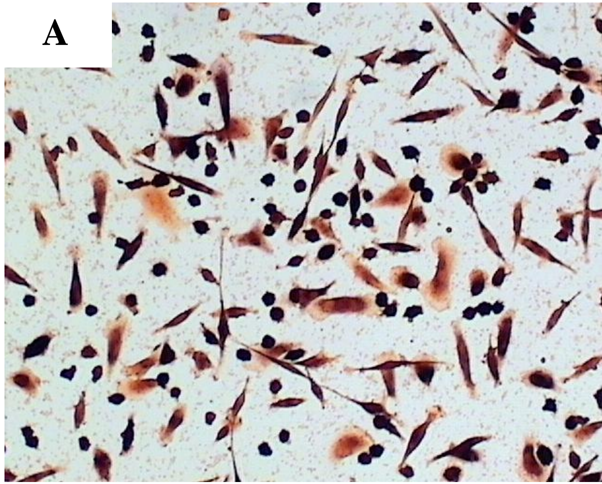
Figure 5.2 Effect of Imatinib on the Morphological Response of GM-CSF Stimulated Monocytes to Lipopolysaccharide Activation.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) in the presence or absence of imatinib for five days. These cultured monocytes were then activated with 1µg/mL lipopolysaccharide (LPS) for 48 hours in the presence of GM-CSF stimulation and imatinib treatment, and stained with naphthol acetate esterase to assess cellular morphology. In the absence of LPS and imatinib treatment (**A**), the differentiation of monocytes was typified by spindle-like morphology. In the presence of LPS (**B**), pseudopodia formation was significantly enhanced, and cells had a spiked morphology. The addition of 0.3µM imatinib (**C**) in LPS activated cultures had a minimal effect on cell morphology. At 1.0µM (**D**), 5.0µM (**E**) and 10.0µM (**F**) imatinib, pseudopodia formation was reduced and the cells were of a more rounded morphology. Photographs were taken at 100 times magnification.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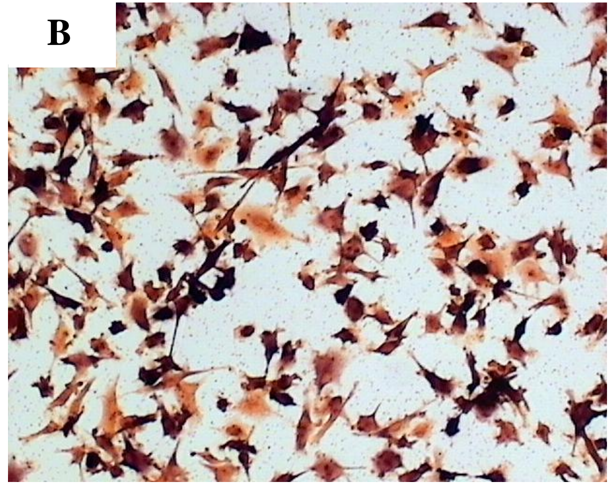
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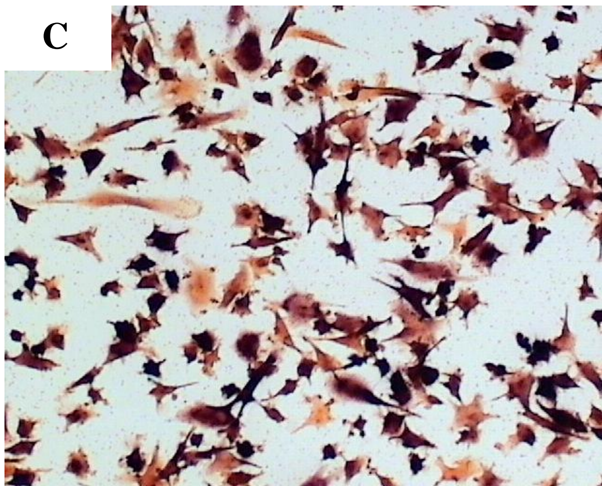
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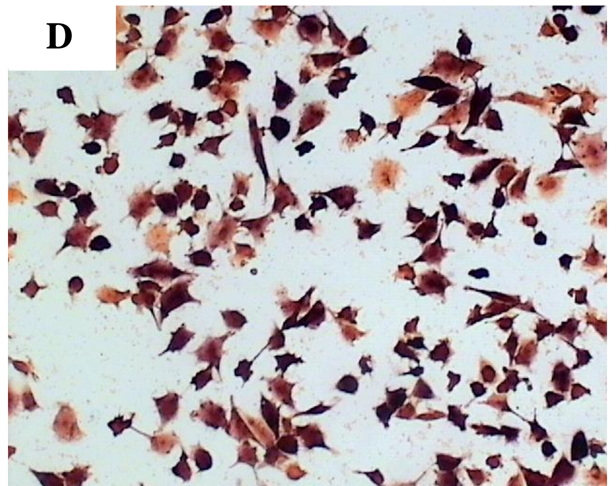
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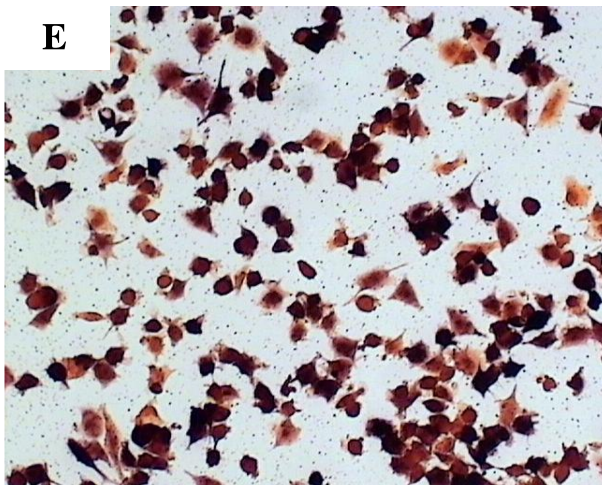
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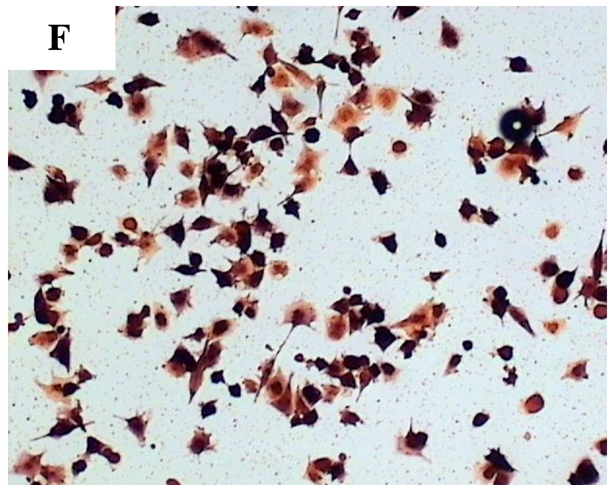
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5.2.1.3 M-CSF & GM-CSF Stimulated Monocytes

LPS treatment of monocytes stimulated with a combination of M-CSF and GM-CSF yielded results that were similar to GM-CSF stimulated monocytes, with LPS enhancing pseudopodia formation (Figure 5.3). The addition of 0.3µM imatinib had little effect on the morphology of LPS activated CM (Figure 5.3C). At 1.0µM imatinib (Figure 5.3D) pseudopodia were shorter in length compared to control cultures (Figure 5.3B), and at 5.0µM (Figure 5.3E) and 10.0µM (Figure 5.3F) imatinib, pseudopodia formation was limited.

5.2.2 Effect of Imatinib on the Production of IL-6 & TNF-α in Response to LPS Activation

To determine whether imatinib affected production of the proinflammatory cytokines IL-6 and TNF-α, CM were activated with LPS in the presence or absence of imatinib, and cytokine production measured at 48 hours.

5.2.2.1 IL-6 Production

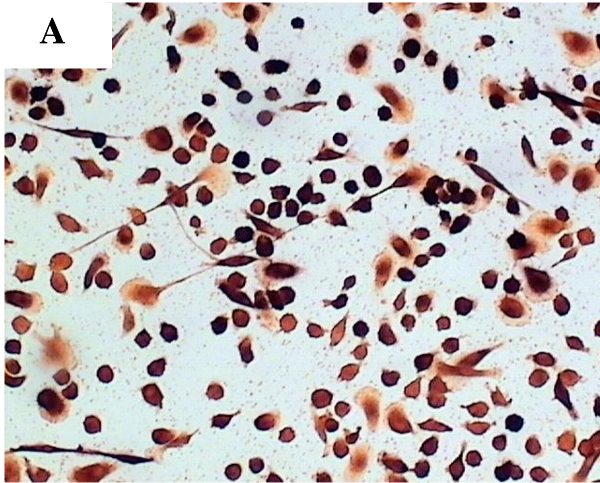
In control cultures, maximal IL-6 production was seen in M-CSF plus GM-CSF stimulated monocyte cultures (approximately 17ng/mL), while minimal IL-6 production was observed in GM-CSF stimulated cultures (Figure 5.4). A dose dependent decrease in IL-6 production in the presence of imatinib was observed in M-CSF stimulated cells from Donors A, B and D and no effect was observed until 5.0µM imatinib in Donor C (Figure 5.4A).

The presence of 0.3µM imatinib had no effect on IL-6 production by GM-CSF stimulated CM from Donors B and C and decreased IL-6 production by 40% in Donor A (Figure 5.4B). At 1.0µM imatinib, reduced IL-6 production was seen in GM-CSF stimulated CM from all donors and at 5.0µM and 10.0µM imatinib, IL-6 production was minimal (Figure 5.4B).

The effect of imatinib on IL-6 production by CM stimulated with a combination of M-CSF and GM-CSF was also examined in response to LPS activation (Figure 5.4C). In the

Figure 5.3 Effect of Imatinib on the Morphological Response of M-CSF and GM-CSF Stimulated Monocytes to Lipopolysaccharide Activation.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with a combination of M-CSF (40ng/mL) and GM-CSF (20ng/mL) in the presence or absence of imatinib for five days. These cultured monocytes were then activated with 1μg/mL lipopolysaccharide (LPS) for 48 hours in the presence of M-CSF and GM-CSF stimulation and imatinib treatment, and stained with naphthol acetate esterase to assess cellular morphology. In the absence of LPS and imatinib treatment (**A**), the differentiation of monocytes into macrophages was typified by spindle-like morphology. In the presence of LPS (**B**), pseudopodia formation was significantly enhanced, and cells had a spiked morphology. The presence of 0.3μM imatinib (**C**) in LPS activated cultures reduced the extent of pseudopodia formation, although the cells still displayed a spiked morphology. At 1.0μM (**D**), 5.0μM (**E**) and 10.0μM (**F**) imatinib, pseudopodia formation was reduced and the cells were of a more rounded morphology. Photographs were taken at 100 times magnification.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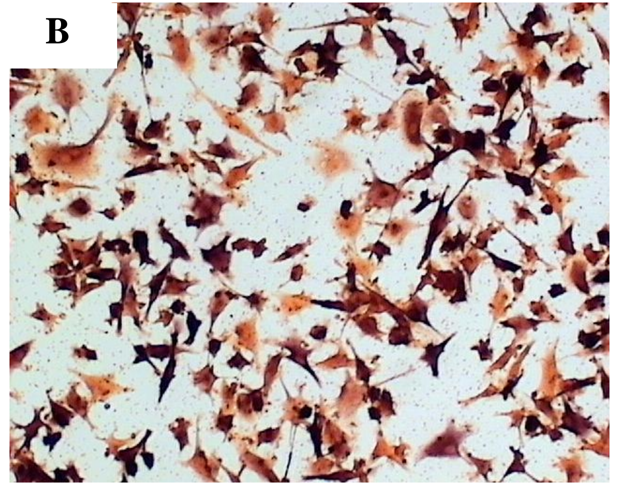
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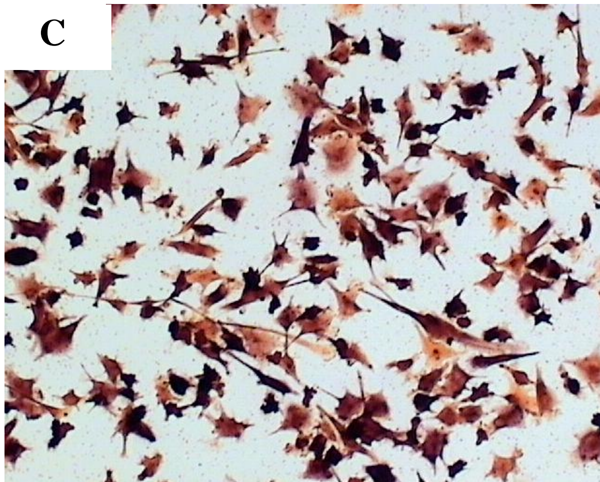
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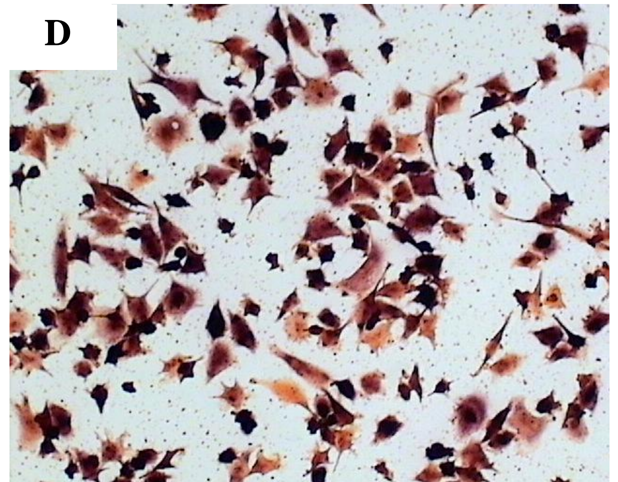
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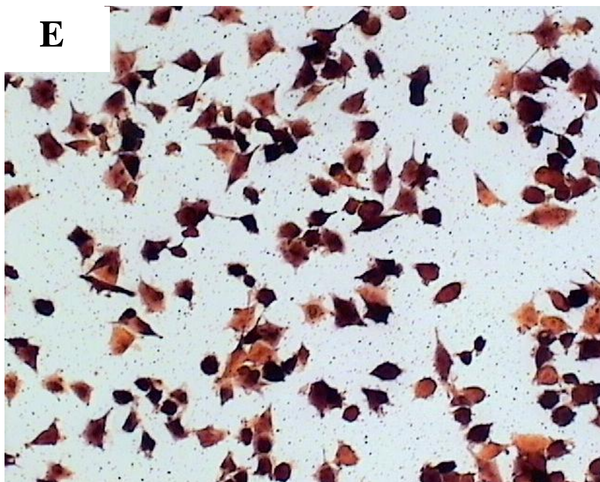
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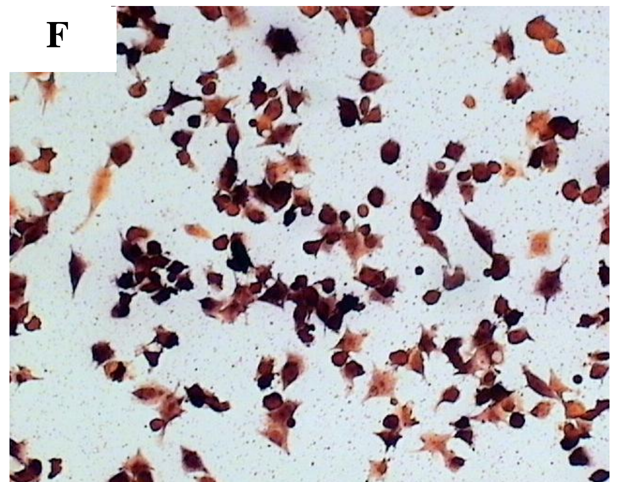
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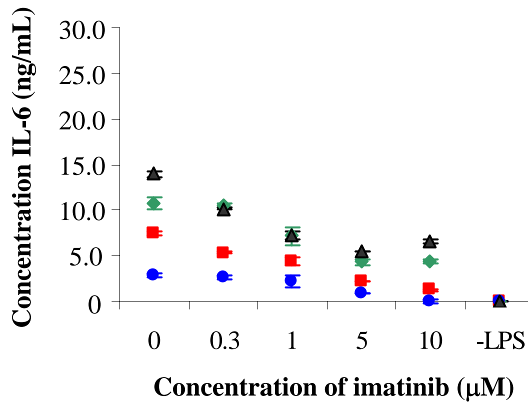
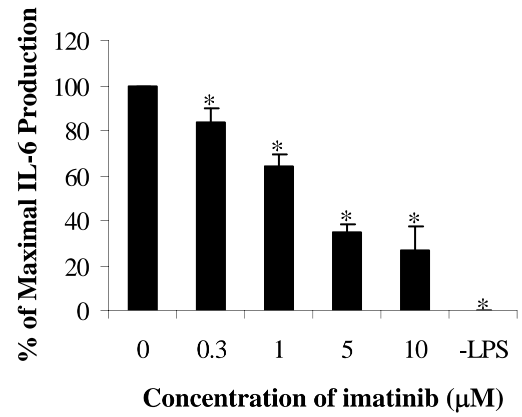
Figure 5.4 Effect of Imatinib on the Production of IL-6 by Cultured Monocytes Activated with Lipopolysaccharide.

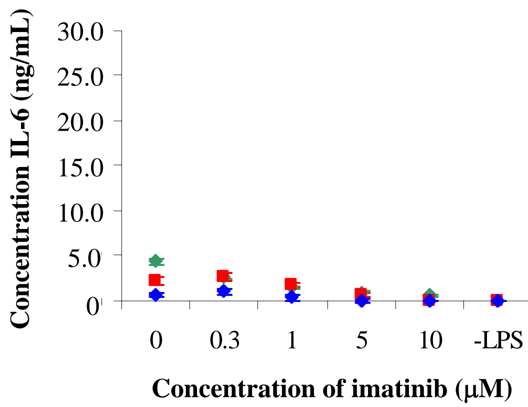
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) (**A**), GM-CSF (20ng/mL) (**B**) or a combination of M-CSF (40ng/mL) and GM-CSF (20ng/mL) (**C**) in the presence or absence of imatinib for five days. Cultures were then activated with 1µg/mL LPS for a period of 48 hours in the presence/absence of imatinib, and supernatants harvested and assayed for IL-6 levels by ELISA. The absorbance value was used to calculate absolute cytokine concentration by extrapolating from a logarithmic line of best fit that was established using known standards. The effect of imatinib on IL-6 production was examined in 6 normal donors (Donor A (◆), Donor B (■), Donor C (●), Donor D (▲), Donor E (◇), Donor F (□)), and all cultures were established in duplicate. A dose response for IL-6 production in the presence of imatinib was seen for each donor stimulated with M-CSF (**A**). Although low levels of IL-6 were produced following stimulation of cultured monocytes with GM-CSF, IL-6 production was still decreased in a dose dependent fashion in the presence of imatinib (**B**). Maximal IL-6 production was observed following stimulation with a combination of M-CSF and GM-CSF, and this was not affected by imatinib at concentrations below 5.0µM (**C**). Normalisation of data relative to untreated controls demonstrated a significant decrease in IL-6 production at concentrations of 0.3µM imatinib and greater for M-CSF stimulated cultures (**A**), 1.0µM imatinib and greater for GM-CSF stimulated cultures (**B**), and 10.0µM imatinib for M-CSF plus GM-CSF stimulated cultures (**C**). Statistical significance was determined relative to untreated controls (* denotes $p < 0.05$).

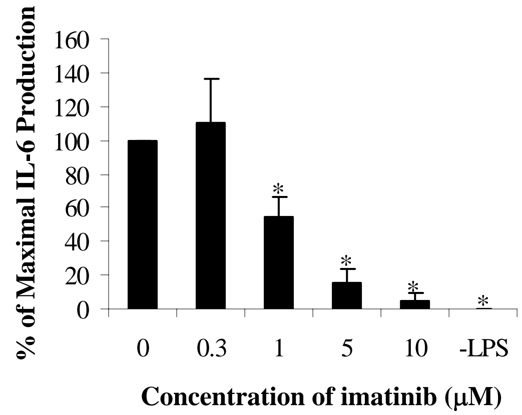
A **M-CSF Stimulated**



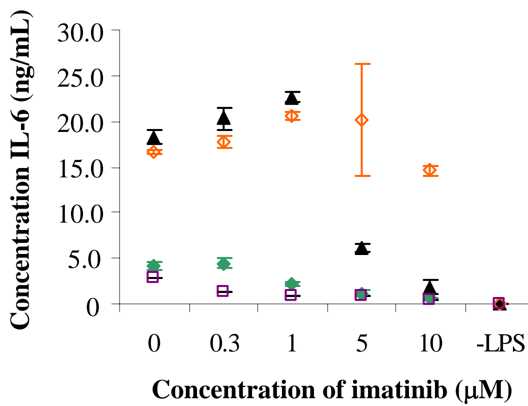


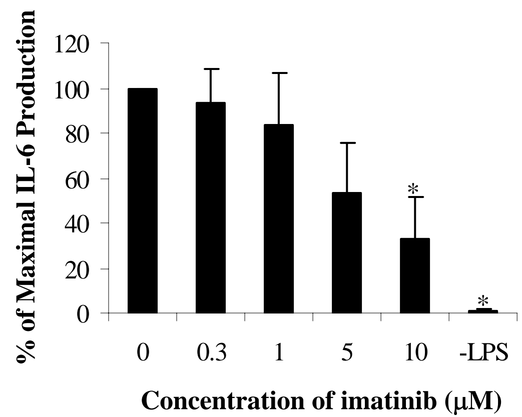
B **GM-CSF Stimulated**





C **M-CSF + GM-CSF Stimulated**





absence of imatinib, cells from Donors A and F produced low levels (<5.0ng/mL) of IL-6, and this was reduced by 50% at concentrations of 1.0μM imatinib and greater. Cells from Donors D and E produced approximately 400% greater levels of IL-6 in the absence of imatinib than Donors A and F, and a 20% decrease in IL-6 production was observed at 5.0μM imatinib in Donor D and a 75% decrease at 10.0μM imatinib in Donor E (Figure 5.4C).

Donor results were normalised relative to untreated controls to facilitate data analysis (Figure 5.4). A dose response relationship was evident in M-CSF stimulated CM following imatinib treatment and IL-6 production was decreased by 15% at 0.3μM imatinib (Figure 5.4A). While M-CSF stimulated CM treated with doses of 5.0μM imatinib or greater had significantly impaired responses to LPS activation, these cells were still capable of IL-6 production as demonstrated by IL-6 levels above unstimulated controls (Figure 5.4A).

GM-CSF stimulated CM were less sensitive to the effect of imatinib than M-CSF stimulated CM, as an imatinib concentration of 1.0μM or greater was required to inhibit IL-6 production by GM-CSF stimulated CM (50% lower than controls, Figure 5.4B). Due to donor variations in the effect of imatinib on IL-6 production in M-CSF plus GM-CSF stimulated CM, a significant decrease in IL-6 production was only observed at 10.0μM imatinib (Figure 5.4C).

5.2.2.2 TNF-α Production

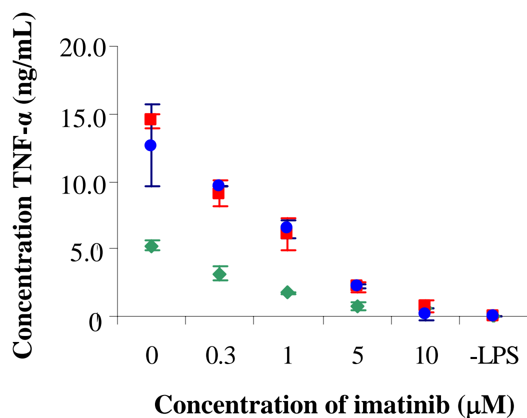
M-CSF stimulated CM produced more TNF-α following activation with LPS (range of 5.0ng/mL to 15.0ng/mL) than CM stimulated with GM-CSF or M-CSF plus GM-CSF (Figure 5.5). A dose dependent decrease in TNF-α production by M-CSF stimulated CM was observed in the presence of imatinib and 0.3μM imatinib reduced TNF-α production by approximately 40% in Donors A and B, and by 25% in Donor C (Figure 5.5A).

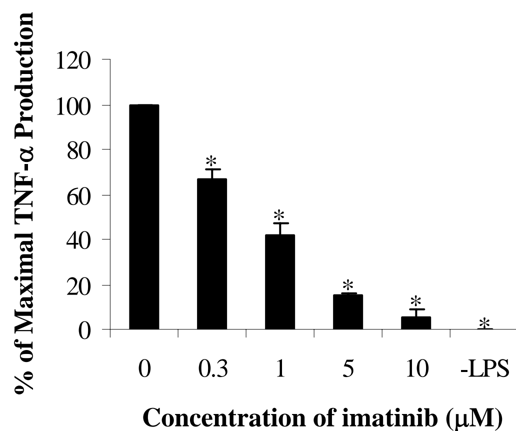
TNF-α production by GM-CSF stimulated CM was 300-400% lower than in CM stimulated with M-CSF (2.0-3.0ng/mL versus 5-15ng/mL) (Figure 5.5B). TNF-α production by GM-CSF stimulated CM was also less affected by imatinib treatment than

Figure 5.5 Effect of Imatinib on the Production of TNF-α by Cultured Monocytes Activated with Lipopolysaccharide.

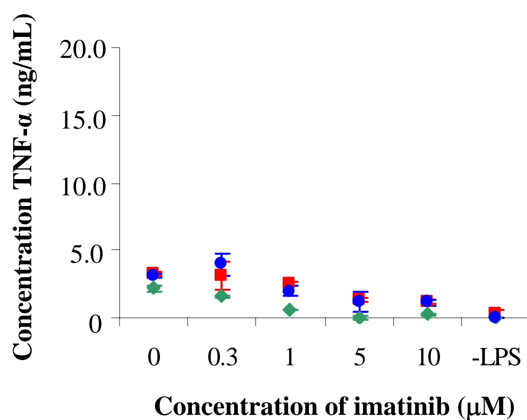
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) (**A**), GM-CSF (20ng/mL) (**B**) or a combination of M-CSF (40ng/mL) and GM-CSF (20ng/mL) (**C**) in the presence or absence of imatinib for five days. Cultures were then activated with 1µg/mL LPS for a period of 48 hours in the presence/absence of imatinib, and supernatants harvested and assayed for TNF-α levels by ELISA. The absorbance value was used to calculate absolute cytokine concentration by extrapolating from a logarithmic line of best fit that was established using known standards. The effect of imatinib on the ability of cultures to produce TNF-α was examined in 6 normal donors (Donor A (◆), Donor B (■), Donor C (●), Donor D (▲), Donor G (○), Donor H (▲)), and all cultures were established in duplicate. Maximal TNF-α production was observed following stimulation of cultures with M-CSF, and a dose response for TNF-α production in the presence of imatinib was seen for Donors A, B and C (**A**). Low levels of TNF-α production were observed following stimulation of cultures with GM-CSF, and were reduced in the presence of 1.0µM imatinib or greater in Donors B and C (**B**). TNF-α production by cultures stimulated with M-CSF plus GM-CSF was reduced in the presence of 1.0µM imatinib for Donor G, and at 5.0µM imatinib in Donors A and H (**C**). Normalisation of data relative to untreated controls demonstrated a significant decrease in TNF-α production at 0.3µM imatinib for M-CSF stimulated cultures (**A**), 1.0µM imatinib for GM-CSF stimulated cultures (**B**), and 5.0µM imatinib for M-CSF plus GM-CSF stimulated cultures (**C**). Statistical significance was determined relative to untreated controls (* denotes $p < 0.05$).

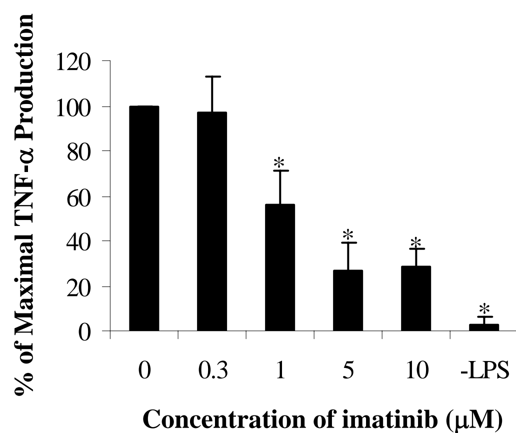
A M-CSF Stimulated



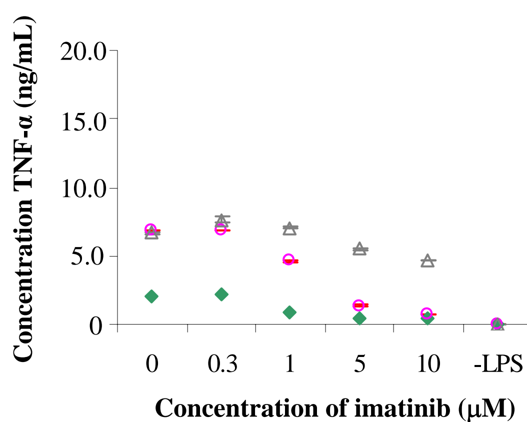


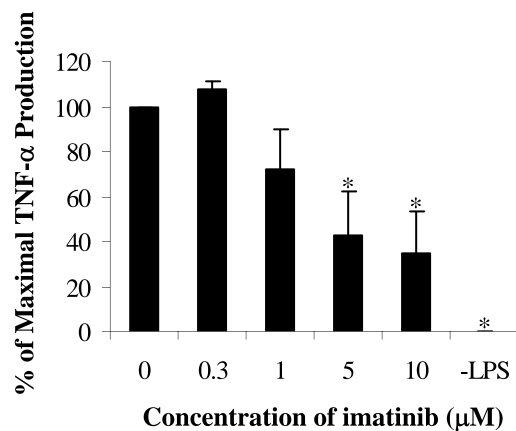
B GM-CSF Stimulated





C M-CSF + GM-CSF Stimulated





M-CSF stimulated CM, as 0.3µM imatinib decreased TNF-α production by 25% in only 1 donor (Donor A). At 1.0µM imatinib, TNF-α production by GM-CSF stimulated CM was reduced by 75% in Donor A and 30% in Donors B and C (Figure 5.5B).

Variation was observed in TNF-α production by M-CSF plus GM-CSF stimulated CM (range of 2.5ng/mL to 7ng/mL) (Figure 5.5C). Similar to GM-CSF stimulated CM, production of TNF-α by M-CSF plus GM-CSF stimulated CM was minimally affected by imatinib treatment at concentrations below 1.0µM (Figure 5.5C).

To determine whether a significant TNF-α response trend could be established following imatinib treatment and to account for patient variability, donor data was normalised relative to untreated controls (Figure 5.5). A dose dependent decrease in TNF-α production in the presence of imatinib was observed in M-CSF stimulated CM and at 0.3µM imatinib, TNF-α production was reduced by approximately 35% relative to untreated controls (Figure 5.5A). GM-CSF and M-CSF plus GM-CSF stimulated CM were less vulnerable to imatinib treatment as 1.0µM imatinib was required to significantly reduce TNF-α production by GM-CSF stimulated CM (Figure 5.5B), and 5.0µM imatinib was required to inhibit TNF-α production by CM stimulated M-CSF plus GM-CSF (Figure 5.5C).

5.2.3 Effect of Imatinib on the Phagocytosis of Zymosan Particles by Cultured Monocytes

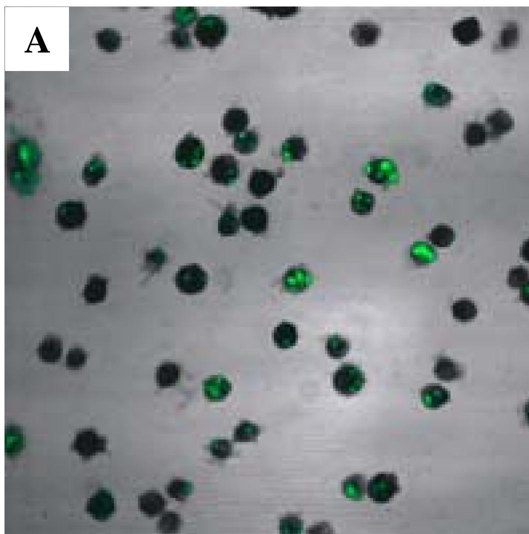
To examine the ability of imatinib treated CM to phagocytose non-opsonised and opsonised zymosan by mannose or Fc receptors respectively, CM were incubated for 1 hour with fluorescently labelled zymosan particles and analysed using confocal microscopy and flow cytometry.

5.2.3.1 Non-Opsonised Zymosan

In Figure 5.6, the effect of imatinib on M-CSF (Figure 5.6A-C) and GM-CSF (Figure 5.6D-F) stimulated CM to phagocytose non-opsonised zymosan was examined using confocal microscopy. In the absence of imatinib treatment (Figures 5.6A and 5.6D), approximately 50-60% of cells contained non-opsonised zymosan particles. Treatment of

Figure 5.6 Effect of Imatinib on the Phagocytosis of Non-Opsonised Zymosan Bioparticles by M-CSF or GM-CSF Stimulated Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) (**A-C**) or GM-CSF (20ng/mL) (**D-F**) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes (CM) were incubated with non-opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of M-CSF or GM-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed zymosan bioparticles, fixed, and stained with naphthol acetate esterase. In the absence of imatinib treatment (**A** and **D**), approximately 50-60% of cells contained non-opsonised zymosan particles. Treatment of CM with 1.0μM imatinib reduced the number of phagocytic cells to 25% and 40% in M-CSF (**B**) and GM-CSF (**E**) stimulated cultures respectively. At 5.0μM imatinib, approximately 6% and 13% of M-CSF (**C**) and GM-CSF (**F**) stimulated CM phagocytosed non-opsonised zymosan respectively. Photographs were taken using a confocal microscope at 200 times magnification.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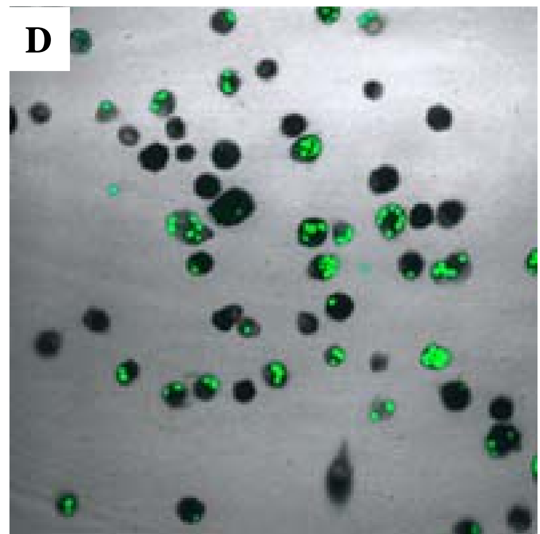
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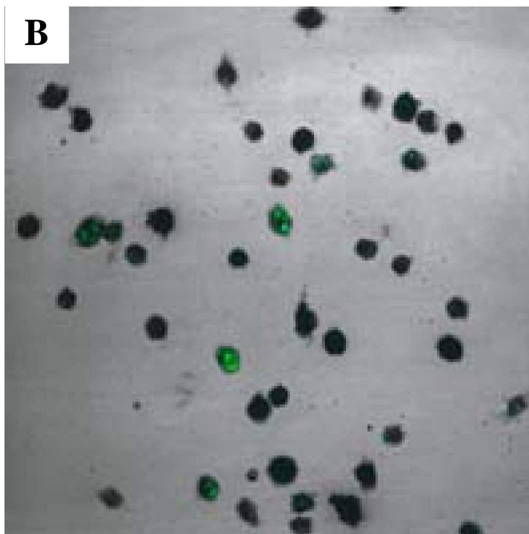
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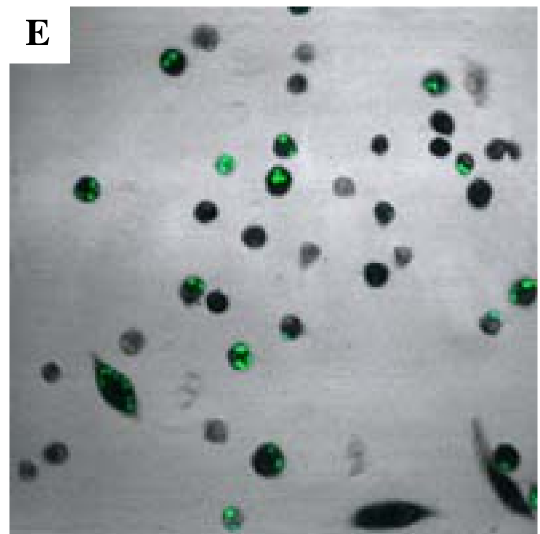
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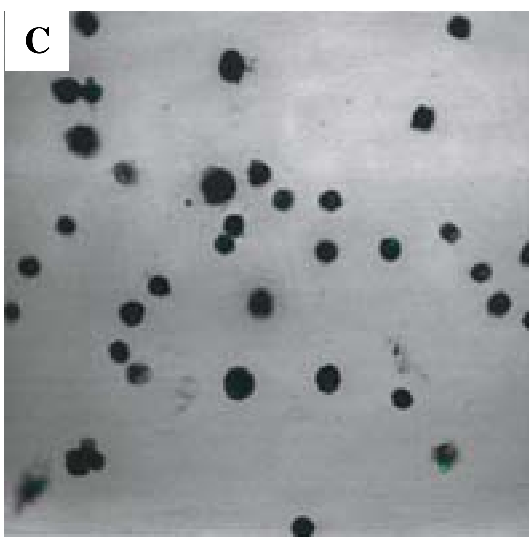
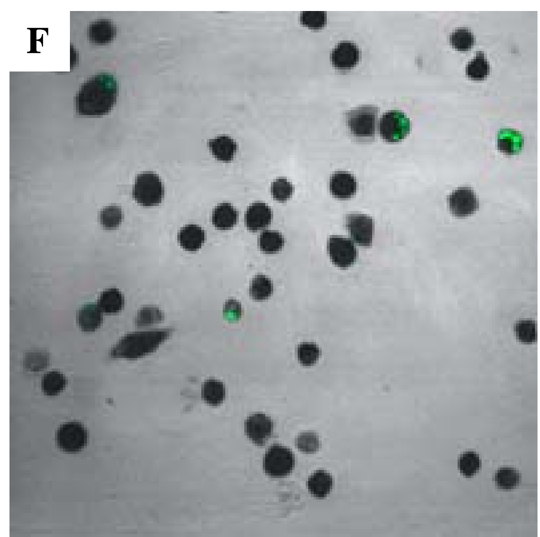

C


F

CM with 1.0μM imatinib reduced the number of phagocytic cells to a greater extent in M-CSF stimulated cultures than GM-CSF stimulated cultures, with approximately 25% and 40% of cells phagocytosing non-opsonised zymosan respectively (Figures 5.6B and 5.6E). At 5.0μM imatinib, M-CSF stimulated CM were also more sensitive to imatinib treatment, as approximately 6% of M-CSF stimulated CM phagocytosed non-opsonised zymosan versus approximately 13% in GM-CSF stimulated CM (Figures 5.6C and 5.6F).

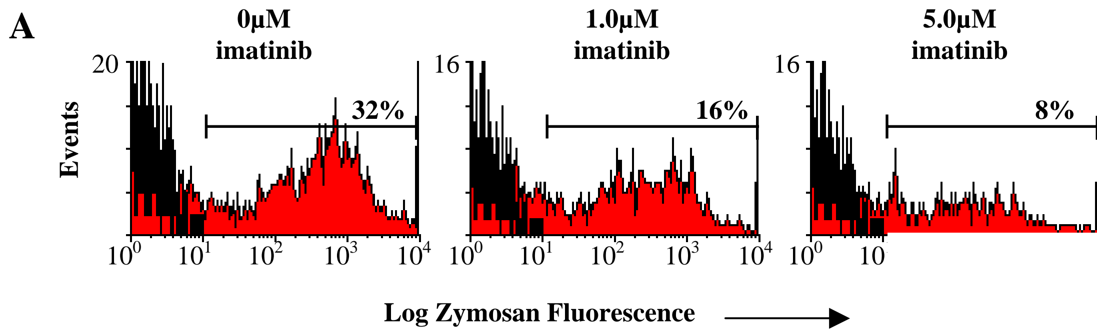
5.2.3.1.1 M-CSF Stimulated Cultures

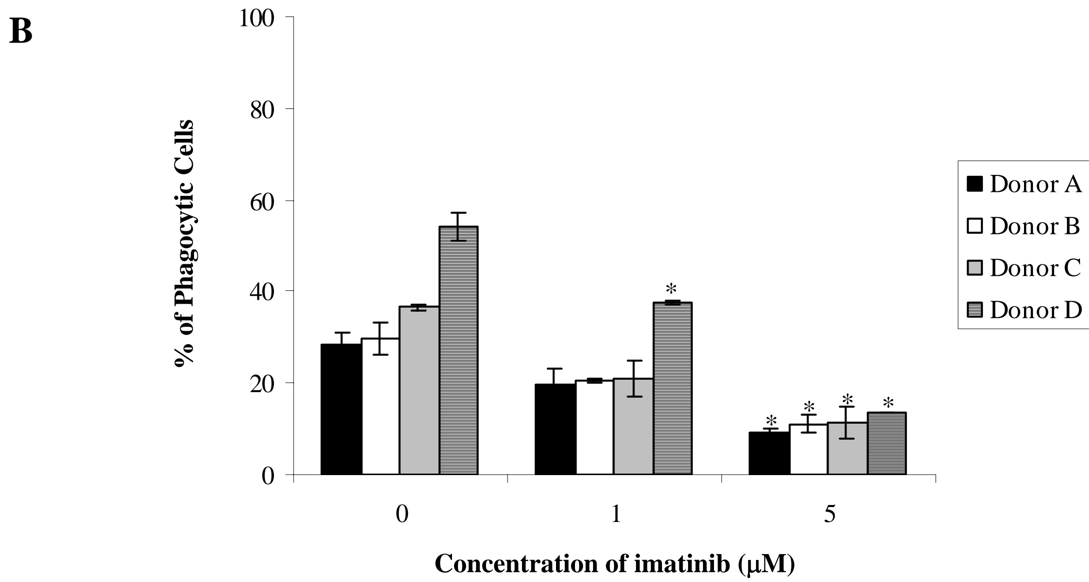
The effect of imatinib on the phagocytosis of non-opsonised zymosan was confirmed using flow cytometry. Example histograms from one M-CSF stimulated donor are shown in Figure 5.7A and display the profile for the uptake of fluorescein-labelled non-opsonised zymosan in the presence or absence of imatinib. In control cultures, 32% of M-CSF stimulated CM increased their fluorescence above background levels, indicating uptake of zymosan. A range of fluorescence values were observed rather than a single defined peak, indicating that the cells took up variable amounts of zymosan. In the presence of 1.0μM or 5.0μM imatinib, the number of cells that phagocytosed non-opsonised zymosan decreased to 16% or 8% respectively, although a range of fluorescence values were still observed. The average amount of zymosan taken up by the cells was not able to be calculated as the fluorescence of some cells was beyond the maximum of the logarithmic scale and skewed mean fluorescence intensity values.

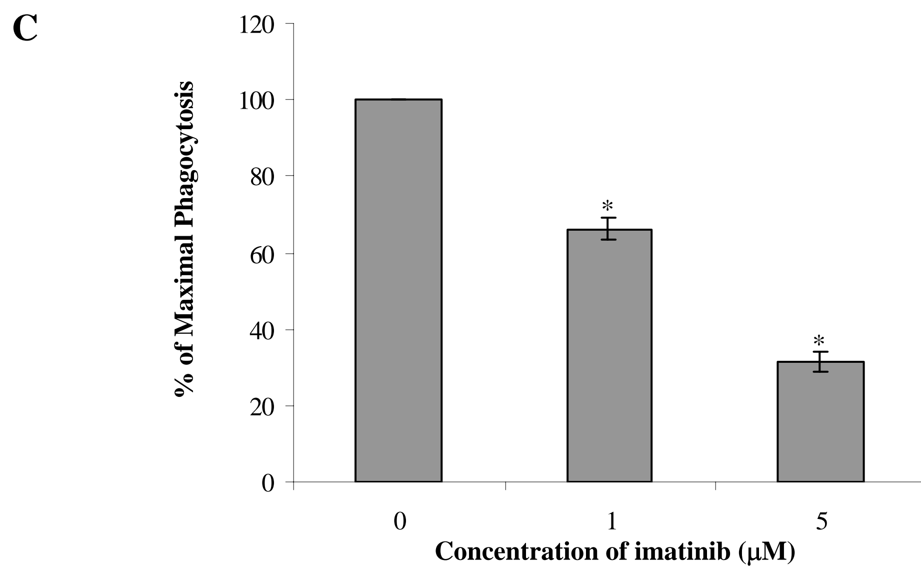
The results from 4 M-CSF stimulated donors are shown in Figure 5.7B. In the absence of imatinib treatment, 29-35% of M-CSF stimulated CM phagocytosed non-opsonised zymosan particles, with the exception of Donor D who demonstrated phagocytic activity in 55% of CM. The addition of 1.0μM imatinib decreased the number of phagocytic cells in all donors by approximately 35%, and this was further reduced to approximately 10% phagocytic cells at 5.0μM imatinib (Figure 5.7B). To determine whether a significant dose response trend could be established following imatinib treatment, data from the 4 donors was normalised relative to untreated controls to account for patient variability (Figure 5.7C). A dose-dependent reduction in the phagocytosis of non-opsonised zymosan by M-CSF stimulated CM was evident and significant at 1.0μM imatinib, where approximately 35% less cells were phagocytic compared to control cultures (Figure 5.7C).

Figure 5.7 Effect of Imatinib on the Phagocytosis of Non-Opsonised Zymosan Bioparticles by M-CSF Stimulated Cultured Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes (CM) were incubated with non-opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of M-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed zymosan bioparticles, and analysed for fluorescence using an analytical Flow Cytometer. Fluorescence histograms derived from a typical donor are displayed (**A**), and demonstrate that 32% of control M-CSF stimulated CM phagocytosed zymosan. Data generated from 4 normal donors is shown in (**B**). The percentage of cells that phagocytosed non-opsonised zymosan was significantly reduced at 1.0μM imatinib in Donor D, and significantly reduced in all donors at 5.0μM imatinib. Normalisation of data relative to untreated controls demonstrated a significant reduction in the percentage of M-CSF stimulated cultured monocytes that phagocytosed non-opsonised zymosan at concentrations of 1.0μM imatinib or greater (**C**). Graphs display mean values ($\pm$SEM) and statistical significance was determined relative to the 0μM imatinib controls (* denotes $p<0.05$). The marker tool was set according to the background fluorescence of cells not incubated with FITC labelled zymosan.


A
0μM imatinib
1.0μM imatinib
5.0μM imatinib
Events
20
16
16
32%
16%
8%
10^0
10^1
10^2
10^3
10^4
10
Log Zymosan Fluorescence


B
% of Phagocytic Cells
100
80
60
40
20
0
Donor A
Donor B
Donor C
Donor D
0
1
5
Concentration of imatinib (μM)


C
% of Maximal Phagocytosis
120
100
80
60
40
20
0
0
1
5
Concentration of imatinib (μM)

5.2.3.1.2 GM-CSF Stimulated Cultures

As observed using confocal microscopy, imatinib inhibited phagocytosis of non-opsonised zymosan by GM-CSF stimulated CM to a lesser extent than M-CSF stimulated CM when cultures were analysed using flow cytometry (Figure 5.8). Flow cytometric data from a typical donor is displayed in Figure 5.8A and demonstrated an increase in fluorescence relative to background values in 37% of GM-CSF stimulated CM in the absence of imatinib, due to uptake of non-opsonised zymosan. As seen in M-CSF stimulated CM, a range of fluorescence values were observed and indicated variation in the cellular uptake of zymosan. In the presence of 1.0μM and 5.0μM imatinib, the number of phagocytic cells was reduced to 29% and 20% respectively. The amount of zymosan particles phagocytosed by these cells did not appear to be affected by imatinib, however this was not able to be confirmed due to the fluorescence of some cells being beyond the maximum of the logarithmic scale (Figure 5.8A).

Greater donor variation was observed in the phagocytosis of non-opsonised zymosan by GM-CSF stimulated CM than M-CSF stimulated CM, with 40-90% of control GM-CSF stimulated CM phagocytosing non-opsonised zymosan (Figure 5.8B). The addition of 1.0μM imatinib significantly decreased phagocytosis in 1 GM-CSF stimulated donor by 30% (Donor E) and at 5.0μM imatinib phagocytosis was 50% lower in Donors A and F, and 90% lower in Donor E compared to controls (Figure 5.8B).

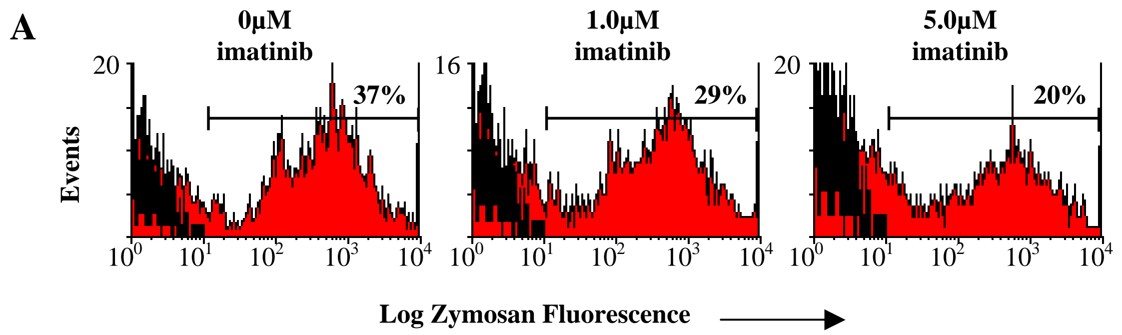
Normalisation of data from GM-CSF stimulated CM revealed that a concentration of 5.0μM imatinib was required to induce a statistically significant decrease of approximately 60% in the phagocytosis of non-opsonised zymosan (Figure 5.8C). This is in contrast to M-CSF stimulated CM where imatinib inhibited phagocytosis of non-opsonised zymosan at 1.0μM or greater.

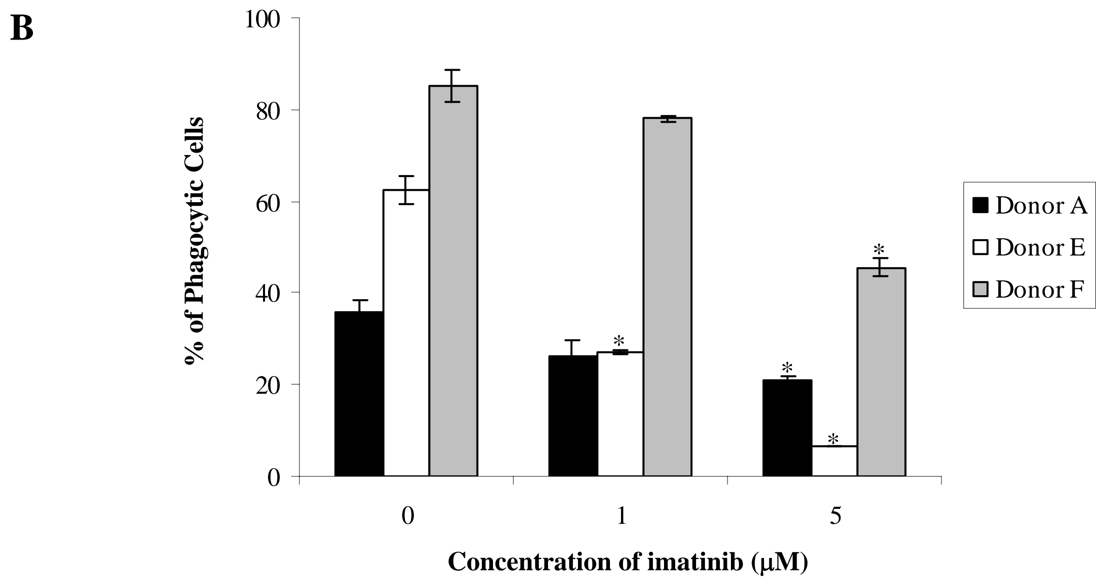
5.2.3.2 Opsonised Zymosan

Confocal microscope images were taken of M-CSF (Figure 5.9A-C) and GM-CSF (Figure 5.9D-F) stimulated CM that had been incubated with opsonised zymosan in the presence or absence of imatinib. In the absence of imatinib treatment (Figure 5.9A and Figure 5.9D), approximately 80% of cells contained FITC-labelled opsonised zymosan particles.

Figure 5.8 Effect of Imatinib on the Phagocytosis of Non-Opsonised Zymosan Bioparticles by GM-CSF Stimulated Cultured Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes were incubated with non-opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of GM-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed zymosan bioparticles, and analysed for fluorescence using an analytical Flow Cytometer. Fluorescence histograms derived from a typical normal donor are displayed (**A**), and demonstrate an increase in fluorescence relative to background values in 37% of control GM-CSF stimulated CM, indicating uptake of non-opsonised zymosan. Data generated from 3 normal donors is shown in (**B**), and demonstrates a significant reduction in the percentage of cells phagocytosing non-opsonised zymosan in the presence 1.0μM imatinib in Donor E, and 5.0μM imatinib in Donors A and F. Normalisation of this data relative to untreated controls demonstrated a significant reduction in the percentage of GM-CSF stimulated CM that phagocytosed non-opsonised zymosan at concentrations of 5.0μM imatinib (**C**). Graphs display mean values ($\pm$SEM) and statistical significance was determined relative to the 0μM imatinib controls (* denotes $p<0.05$). The marker tool was set according to the background fluorescence of cells not incubated with FITC labelled zymosan.


A
0µM
imatinib
1.0µM
imatinib
5.0µM
imatinib
Events
37%
29%
20%
Log Zymosan Fluorescence


B
% of Phagocytic Cells
Donor A
Donor E
Donor F
Concentration of imatinib (µM)

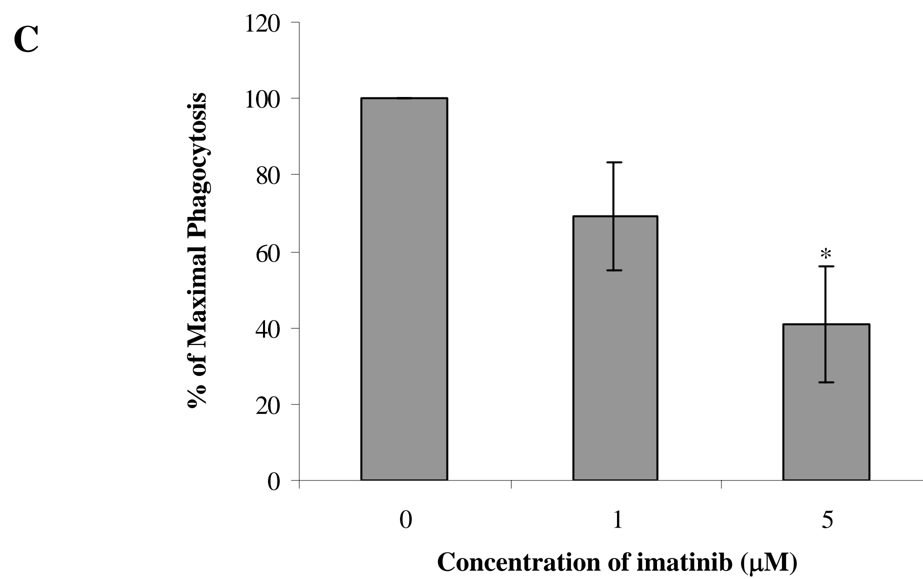
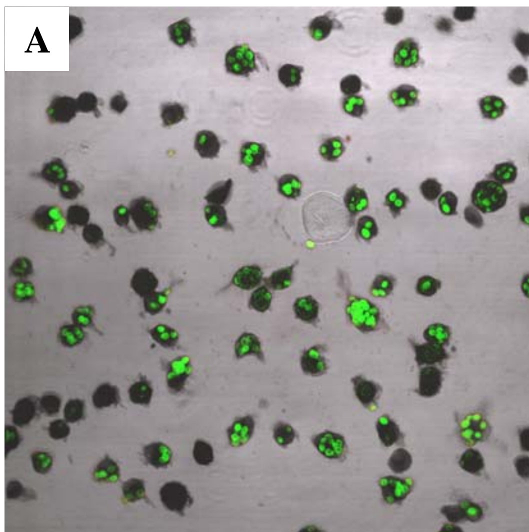
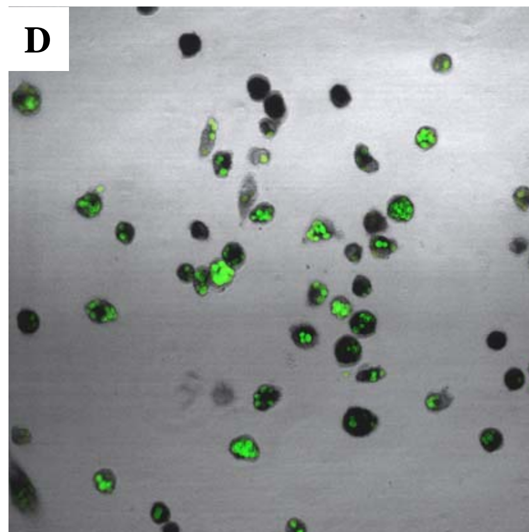
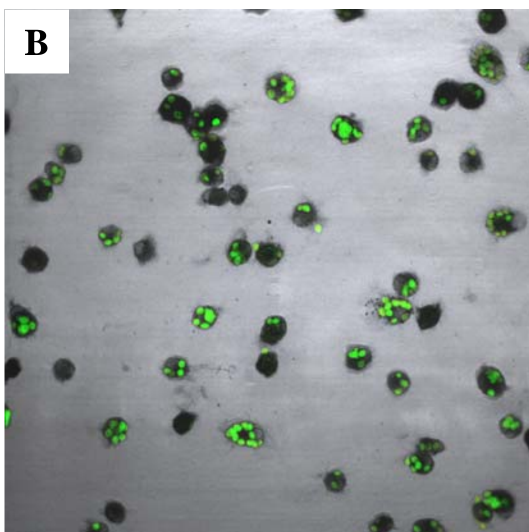
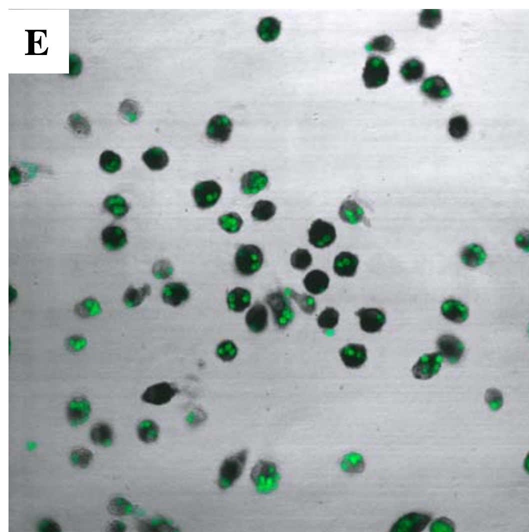
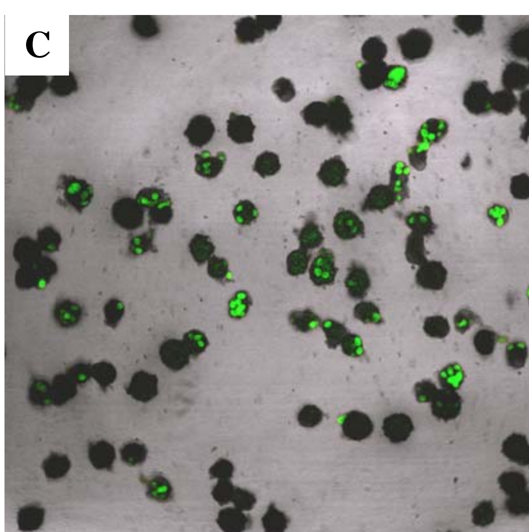
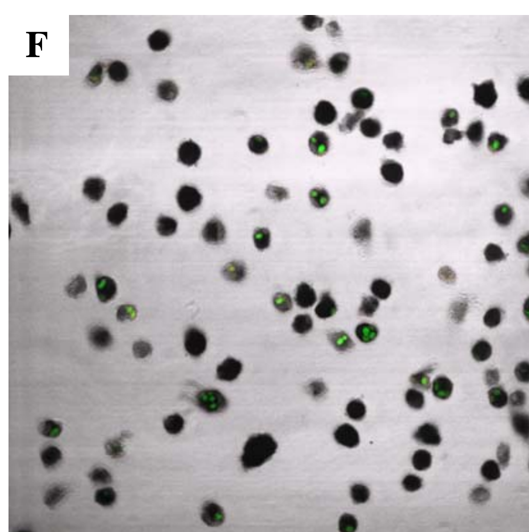

C
% of Maximal Phagocytosis
Concentration of imatinib (µM)

Figure 5.9 Effect of Imatinib on the Phagocytosis of Opsonised Zymosan Bioparticles by M-CSF or GM-CSF Stimulated Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) (**A-C**) or GM-CSF (20ng/mL) (**D-F**) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes were incubated with opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of M-CSF or GM-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed zymosan bioparticles, fixed, and stained with naphthol acetate esterase. In the absence of imatinib treatment (**A** and **D**), approximately 80% of cells contained FITC-labelled opsonised zymosan particles. No reduction in the number of cells that phagocytosed zymosan bioparticles was seen at 1.0μM imatinib (**B** and **E**), while at 5.0μM imatinib, approximately 40% of cells contained zymosan bioparticles (**C** and **F**). Photographs were taken using a confocal microscope at 200 times magnification.


A

D

B

E

C

F

Stimulation of CM in the presence of 1.0μM imatinib had no effect on the number of cells that phagocytosed opsonised FITC-zymosan, in both M-CSF (Figure 5.9B) and GM-CSF (Figure 5.9E) stimulated cultures. At 5.0μM imatinib, approximately 45% of M-CSF stimulated CM contained opsonised FITC-zymosan (Figure 5.9C) and approximately 35% of GM-CSF stimulated CM were phagocytic (Figures 5.9F).

5.2.3.2.1 M-CSF Stimulated Cultures

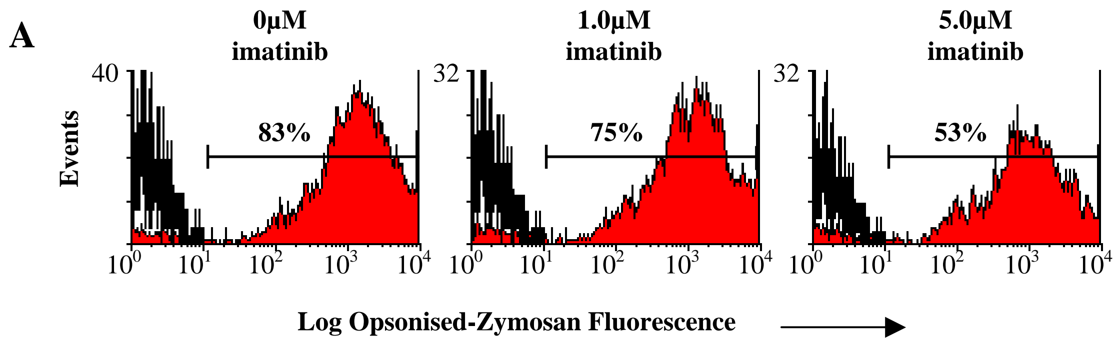
An example of flow cytometric histograms from M-CSF stimulated CM incubated with opsonised zymosan are displayed in Figure 5.10A. In control cultures, 83% of cells increased their fluorescence relative to background levels, indicating that a greater number of M-CSF stimulated CM were able to uptake opsonised zymosan than non-opsonised zymosan. A range of fluorescence intensities was also observed, indicating that cells took up variable amounts of opsonised zymosan. In the presence of 1.0μM and 5.0μM imatinib, the percentage of cells that phagocytosed opsonised zymosan decreased to 75% and 53% respectively. While the fluorescence profile of cells that phagocytosed zymosan appeared to be similar in the presence or absence of imatinib, mean fluorescence intensities were not analysed due to skewing of the MFI by cells beyond the maximum of the logarithmic scale.

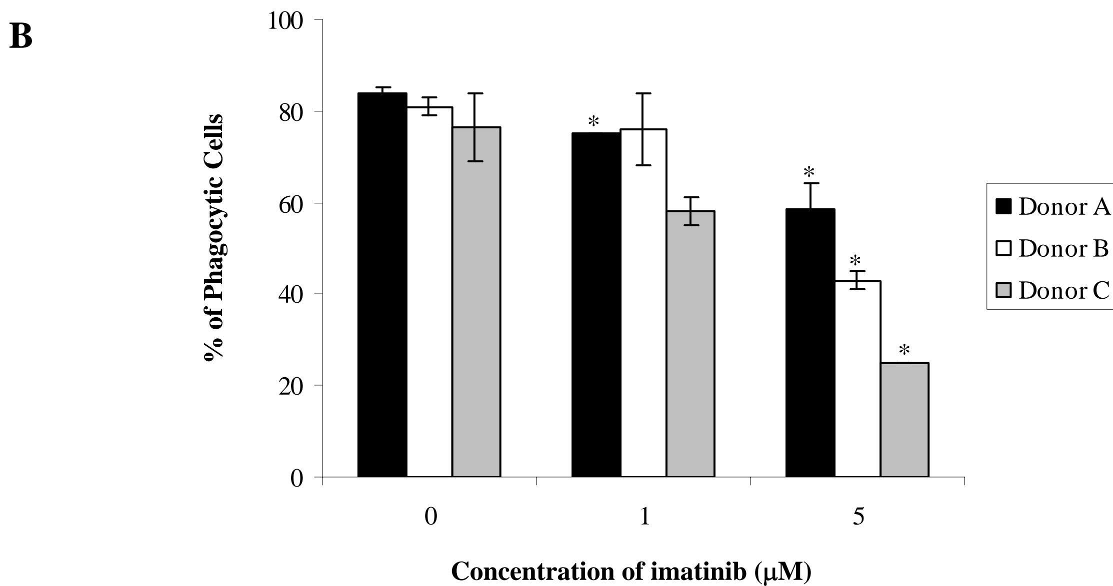
Data from 3 donors was analysed graphically and showed that in the absence of imatinib treatment, 75-85% of M-CSF stimulated CM phagocytosed opsonised zymosan particles (Figure 5.10B). This was similar to the number of phagocytic cells observed using confocal microscopy and these results confirmed that opsonisation of zymosan induced phagocytosis in a greater number of M-CSF stimulated CM compared to non-opsonised zymosan. The addition of 1.0μM imatinib significantly decreased the number of cells that phagocytosed opsonised zymosan by 10% in Donor A, and while a decrease was also observed in Donors B and C, these were not found to be significant (Figure 5.10B). At 5.0μM imatinib, the number of cells that phagocytosed opsonised zymosan was significantly decreased by 40-70% in all donors (Figure 5.10B).

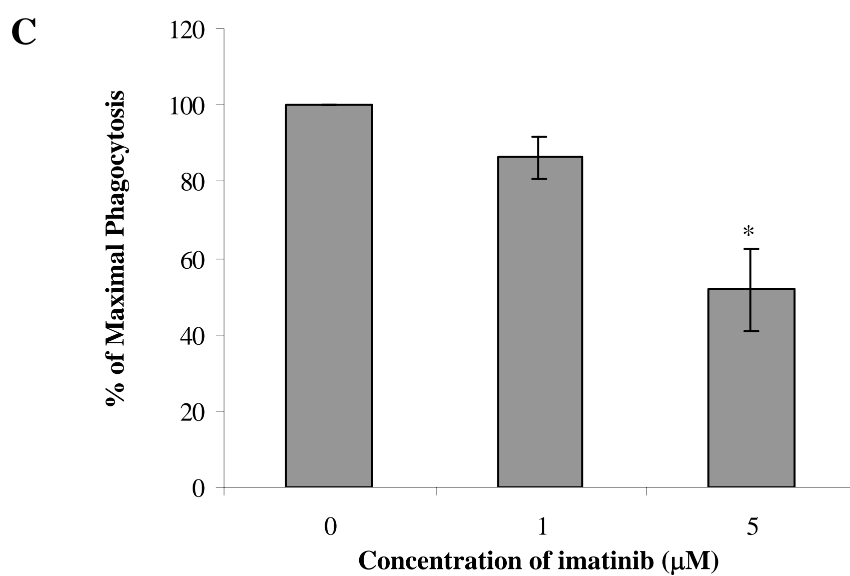
Normalisation of the data relative to untreated controls demonstrated that at 1.0μM imatinib, no significant difference was observed in the number of M-CSF stimulated CM that phagocytosed opsonised zymosan (Figure 5.10C). At 5.0μM imatinib, the number of

Figure 5.10 Effect of Imatinib on the Phagocytosis of Opsonised Zymosan Bioparticles by M-CSF Stimulated Cultured Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes were incubated with opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of M-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed zymosan bioparticles, and analysed for fluorescence using an analytical Flow Cytometer. Fluorescence histograms derived from a typical normal donor are displayed (**A**), and demonstrate that 83% of cells in control cultures increased their fluorescence relative to background levels. Data generated from 3 normal donors is shown in (**B**), and demonstrated a significant reduction in the percentage of cells that phagocytosed opsonised zymosan at 1.0μM imatinib in Donor A, and at 5.0μM imatinib in Donors B and C. Normalisation of data relative to untreated controls demonstrated a 50% decrease in phagocytosis at 5.0μM imatinib (**C**). Graphs display mean values ($\pm$SEM) and statistical significance was determined relative to the 0μM imatinib controls (* denotes $p<0.05$). The marker tool was set according to the background fluorescence of cells not incubated with FITC labelled zymosan.


A
0µM
imatinib
1.0µM
imatinib
5.0µM
imatinib
Events
40
32
32
83%
75%
53%
Log Opsonised-Zymosan Fluorescence


B
100
80
60
40
20
0
% of Phagocytic Cells
0
1
5
Concentration of imatinib (µM)
Donor A
Donor B
Donor C


C
120
100
80
60
40
20
0
% of Maximal Phagocytosis
0
1
5
Concentration of imatinib (µM)

M-CSF stimulated CM that phagocytosed opsonised zymosan was reduced by approximately 50% (Figure 5.10C), confirming the result from confocal microscopy.

5.2.3.2.2 GM-CSF Stimulated Cultures

An example of flow cytometric data from GM-CSF stimulated CM is displayed in Figure 5.11A. A greater number of GM-CSF stimulated CM phagocytosed opsonised zymosan than non-opsonised zymosan with 75% of cells increasing their fluorescence relative to background levels. The addition of imatinib to cultures reduced the number of phagocytic cells to 68% at 1.0μM imatinib and to 54% at 5.0μM imatinib.

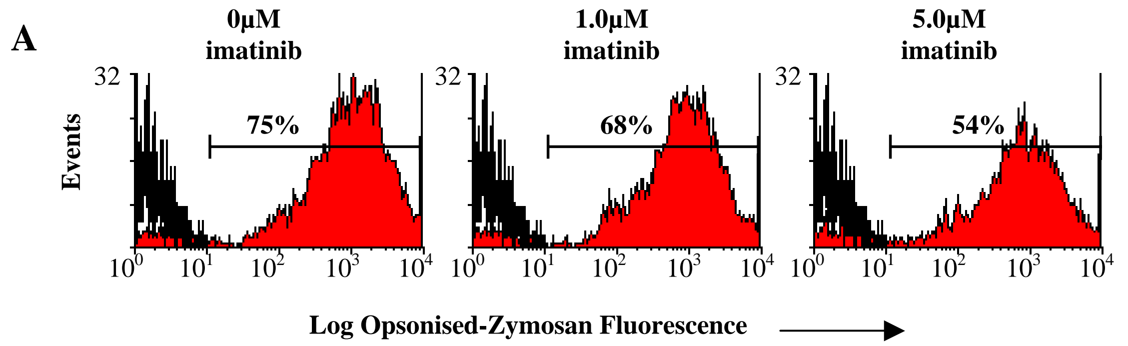
The effect of imatinib on the phagocytosis of opsonised zymosan by GM-CSF stimulated CM in 3 normal donors is displayed in Figure 5.11B. Similar percentages of cells phagocytosed opsonised zymosan in all donors (70%), and the addition of 1.0μM imatinib to cultures had little effect on the number of cells phagocytosing opsonised zymosan (Figure 5.11B). At 5.0μM imatinib, the number of cells phagocytosing opsonised zymosan was reduced by 20-35% in all donors (Figure 5.11B). The absence of an effect of imatinib on phagocytosis of opsonised zymosan particles until 5.0μM imatinib was confirmed following normalisation of results (Figure 5.11C). While this data confirms the observation that phagocytosis of opsonised zymosan by GM-CSF stimulated CM is affected by imatinib concentrations of 5.0μM, the 60% reduction observed using flow cytometry was greater than the 25% reduction observed with confocal microscopy. This may be attributable to the higher sensitivity achieved using flow cytometry.

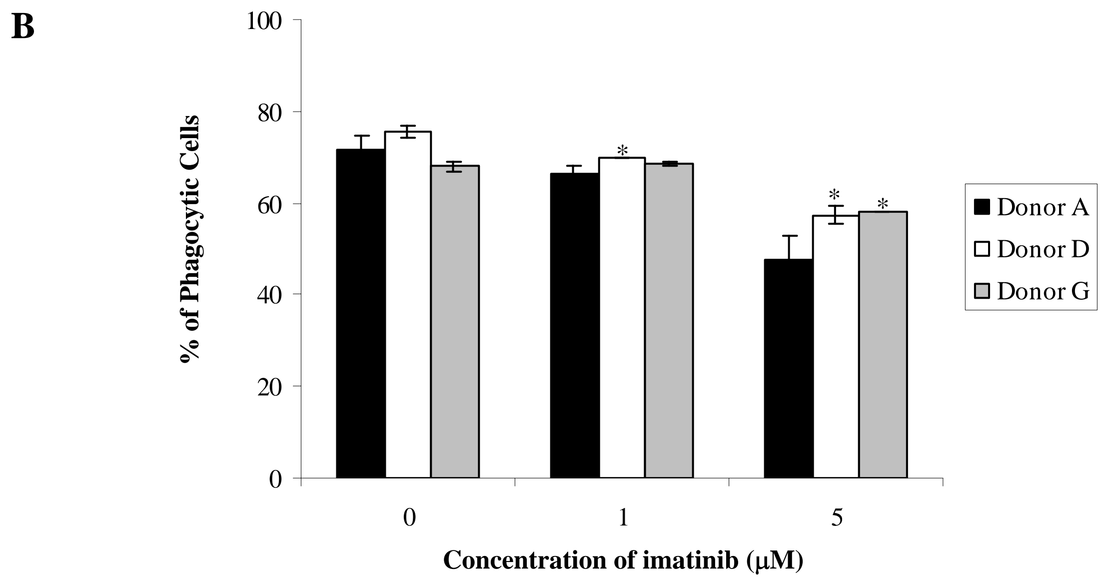
5.2.4 Effect of Imatinib on the Ability of Cultured Monocytes to Stimulate a Mixed Lymphocyte Reaction

Having observed that treatment of CM, and in particular M-CSF stimulated CM, with imatinib impaired their ability to phagocytose and respond to LPS activation, the next question to address was whether these cells were also impaired in their ability to induce proliferation in a mixed lymphocyte reaction (MLR) (Figure 5.12 and 5.13). Monocyte cultures that had been stimulated with M-CSF or GM-CSF for a period of 5 days in the presence or absence of imatinib were washed thoroughly, and used to stimulate a responder cell population that was enriched for T cells. CFSE labelled T cells were incubated with

Figure 5.11 Effect of Imatinib on the Phagocytosis of Opsonised Zymosan Bioparticles by GM-CSF Stimulated Cultured Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) in the presence or absence of imatinib for a period of 7 days. These cultured monocytes were incubated with opsonised FITC-labelled zymosan bioparticles for 1 hour in the presence of GM-CSF stimulation and imatinib treatment. Cells were then extensively trypsinised to detach non-phagocytosed opsonised zymosan bioparticles, and analysed for fluorescence using an analytical Flow Cytometer. Fluorescence histograms derived from a typical normal donor are displayed (**A**), and demonstrate that 75% of cells in control cultures increased their fluorescence relative to background levels. Data generated from 3 normal donors is shown in (**B**), and demonstrates a significant reduction in the percentage of cells phagocytosing opsonised zymosan at 1.0μM imatinib in Donor D, and at 5.0μM imatinib in Donors A and G. Normalisation of the data relative to untreated controls demonstrated a 22% decrease in phagocytosis at 5.0μM imatinib (**C**). Graphs display mean values ($\pm$SEM) and statistical significance was determined relative to the 0μM imatinib controls (* denotes $p<0.05$). The marker tool was set according to the background fluorescence of cells not incubated with FITC labelled zymosan.


A
0µM imatinib
1.0µM imatinib
5.0µM imatinib
32
Events
75%
68%
54%
Log Opsonised-Zymosan Fluorescence


B
% of Phagocytic Cells
Donor A
Donor D
Donor G
Concentration of imatinib (µM)

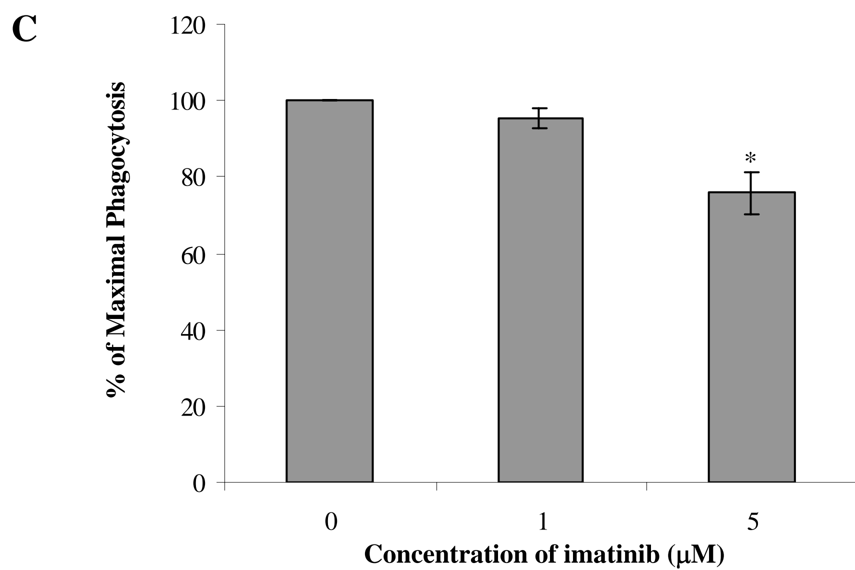
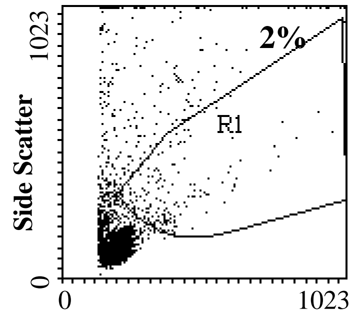
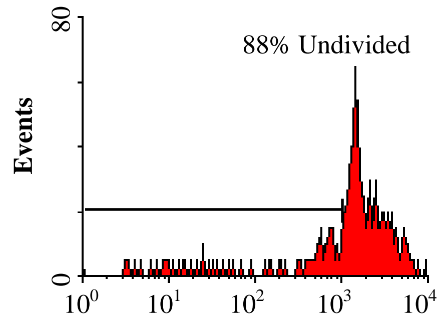

C
% of Maximal Phagocytosis
Concentration of imatinib (µM)

Figure 5.12 Effect of Imatinib on the Ability of M-CSF Stimulated Monocyte Cultures to Stimulate a Mixed Lymphocyte Reaction.

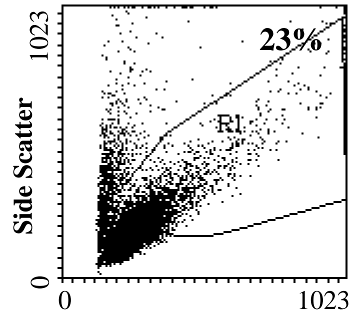
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) in the presence or absence of imatinib for a period of 5 days. The resultant cells were washed thoroughly to remove all traces of the drug, and incubated with CFSE-stained nylon wool purified responder cells for a further 5 days. The non-adherent cell fraction that constituted responder cells was then harvested and analysed for CFSE fluorescence on an analytical Flow Cytometer. Resultant dot-plots and histograms generated from the data are displayed, and controls include unstimulated CFSE-labelled responder cells ("No Stimulus"), and Concavalin A stimulated CFSE-labelled responder cells. When M-CSF stimulated CM were not treated with imatinib, 16% of responder cells fell into the activated cell region, R1, and of these cells, 50% remained undivided. Treatment of CM with imatinib reduced the percentage of responder cells that fell into region R1, and increased the number of these cells that remained undivided. Histogram plots represent data from cells falling into the R1 gate indicated on dot plots. Results are representative of data derived from three individual experiments using different donors. Markers were set according to the maximum fluorescence of undivided, non-stimulated controls.

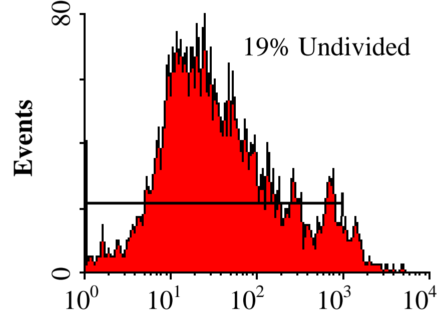
No Stimulus



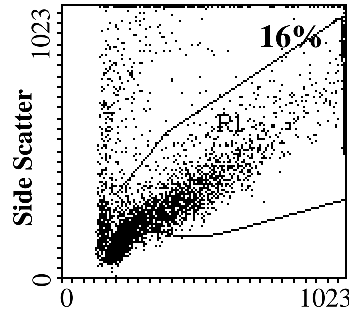


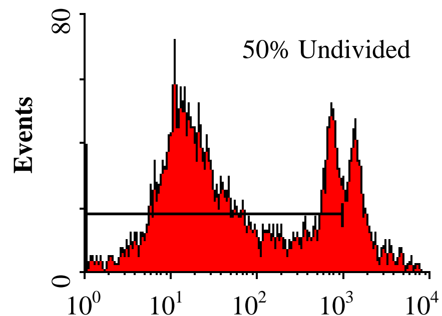
Concavalin A



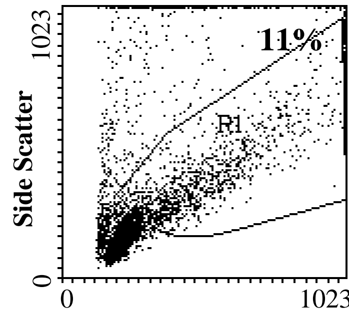


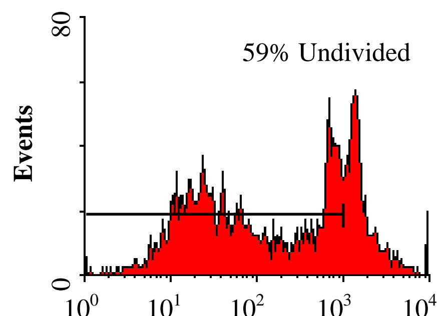
0μM imatinib



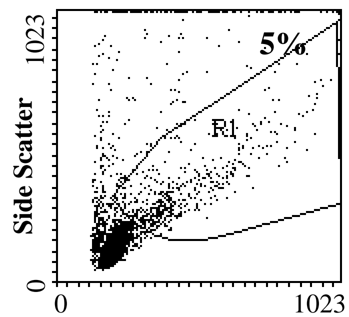


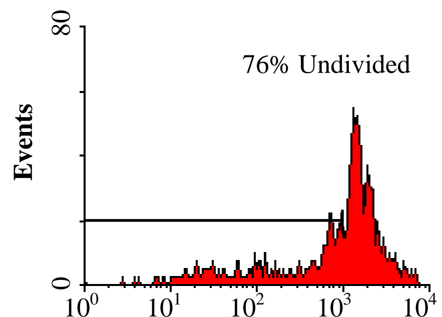
1.0μM imatinib



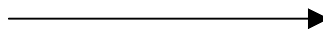


5.0μM imatinib





Forward Scatter



CFSE Fluorescence

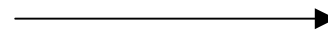
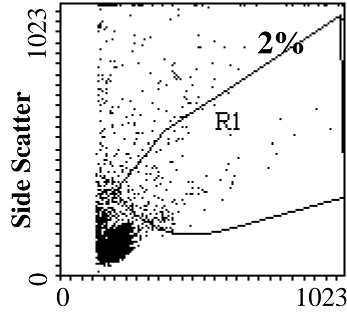
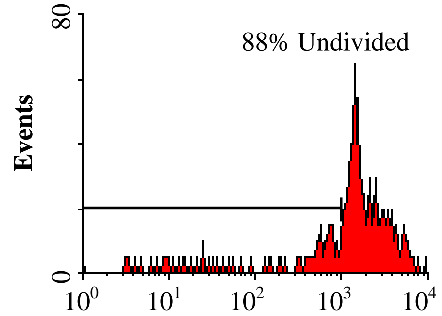


Figure 5.13 Effect of Imatinib on the Ability of GM-CSF Stimulated Monocyte Cultures to Stimulate a Mixed Lymphocyte Reaction.

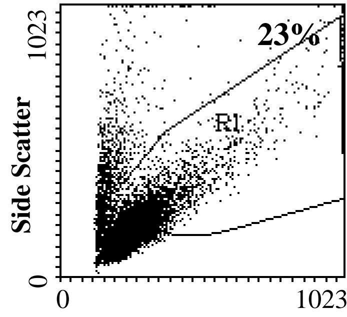
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) in the presence or absence of imatinib for a period of 5 days. The resultant cells were washed thoroughly to remove all traces of the drug, and incubated with CFSE-stained nylon wool purified responder cells for a further 5 days. The non-adherent cell fraction that constituted responder cells was then harvested and analysed for CFSE fluorescence on an analytical Flow Cytometer. Resultant dot-plots and histograms generated from the data are displayed, and controls include unstimulated CFSE-labelled responder cells ("No Stimulus"), and Concavalin A stimulated CFSE-labelled responder cells. When GM-CSF stimulated CM were not treated with imatinib, 7% of cells fell into the activated cell region R1 indicating that GM-CSF stimulated monocytes were unable to stimulate marked cell division. This was also observed in monocyte cultures that had been treated with 1.0μM and 5.0μM imatinib. Histogram plots represent data from cells falling into the R1 gate indicated on dot plots. Results are representative of data derived from three individual experiments using different donors. Markers were set according to the maximum fluorescence of undivided, non-stimulated controls.

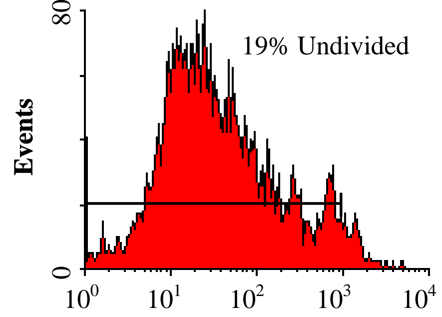
No Stimulus



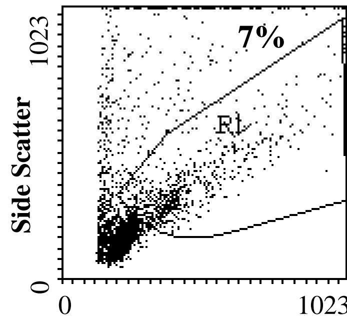


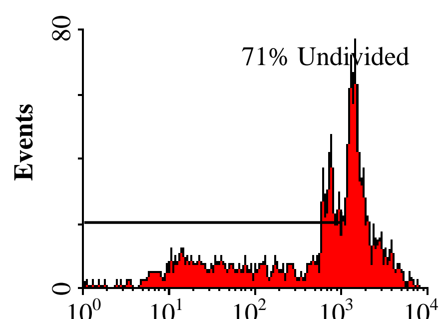
Concavalin A



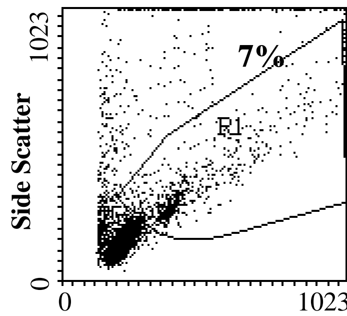


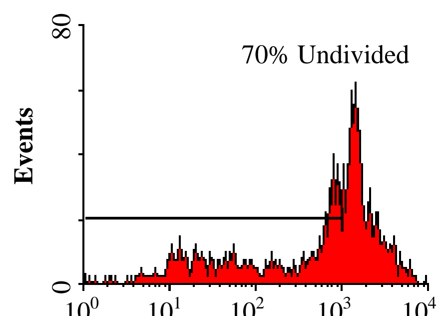
0μM imatinib



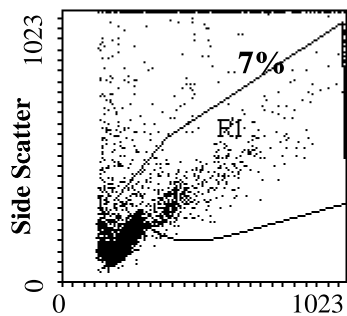


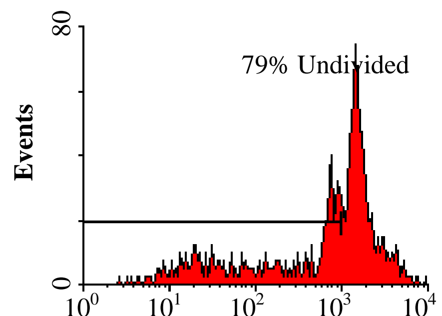
1.0μM imatinib



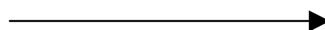


5.0μM imatinib

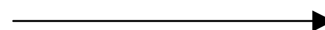




Forward Scatter



CFSE Fluorescence



CM for a period of 5 days, after which time the non-adherent fraction was harvested and analysed for CFSE fluorescence using flow cytometry. The activation of responder T cells was determined by the appearance of CFSE labelled cells within a gate defining blast cells, and dividing cells were identified within this gate by a reduction in CFSE fluorescence. The percentage of starting cells that remained undivided was calculated according to the equation in section 2.3.6.2.

5.2.4.1 M-CSF Stimulated Monocyte Cultures

When control M-CSF stimulated CM were used to stimulate nylon wool-enriched responder cells, 16% of responder cells fell into the blast cell gate, and of these gated cells, 50% had undergone cell division (Figure 5.12). In the absence of stimulating cells, only 2% of responder cells fell into the blast cell gate, and of these cells, 12% underwent cell division. Activation of responder cells with Concavalin A resulted in 23% of cells falling into the blast cell gate, with 81% of gated starting cells having undergone cell division by the end of the culture (Figure 5.12).

M-CSF stimulated monocytes that had been cultured in the presence of 1.0μM imatinib induced division in 18% less starting responder cells when compared to control cultures, and 30% less cells fell into the blast cell gate (Figure 5.12). When the M-CSF stimulated CM had been cultured in the presence of 5.0μM imatinib, the percentage of gated starting cells undergoing division was 5%, demonstrating that these CM were less able to stimulate division in the responder cells (Figure 5.12). This decrease in the percentage of cells undergoing division in the presence of imatinib occurred concomitantly with a decrease in the percentage of cells falling into the blast cell gate, as only 5% of cells fell into this gate at 5.0μM imatinib versus 16% in control cultures.

5.2.4.2 GM-CSF Stimulated Monocyte Cultures

GM-CSF stimulated CM were unable to stimulate similar levels of responder cell division, regardless of whether the cells were grown in cultures with or without imatinib (Figure 5.13). In control cultures where GM-CSF stimulated CM had not been treated with imatinib, 29% of gated starting responder cells underwent cell division. When GM-CSF

stimulated CM were pre-treated with 5.0μM imatinib, 21% of starting responder cells were induced to divide.

Although GM-CSF stimulated CM appeared to have reduced capacity to stimulate division in responder cells in an MLR, the CFSE profile of these cells was different to the no-stimulus control. This can be attributed to 5% more responder cells falling into the blast cell gate following their co-culture with GM-CSF stimulated CM and suggests that the responder cells were stimulated to increase their forward and side scatter in the absence of promoting proliferation (Figure 5.13)

5.2.5 Effect of Imatinib on CD80 & CD86 Expression

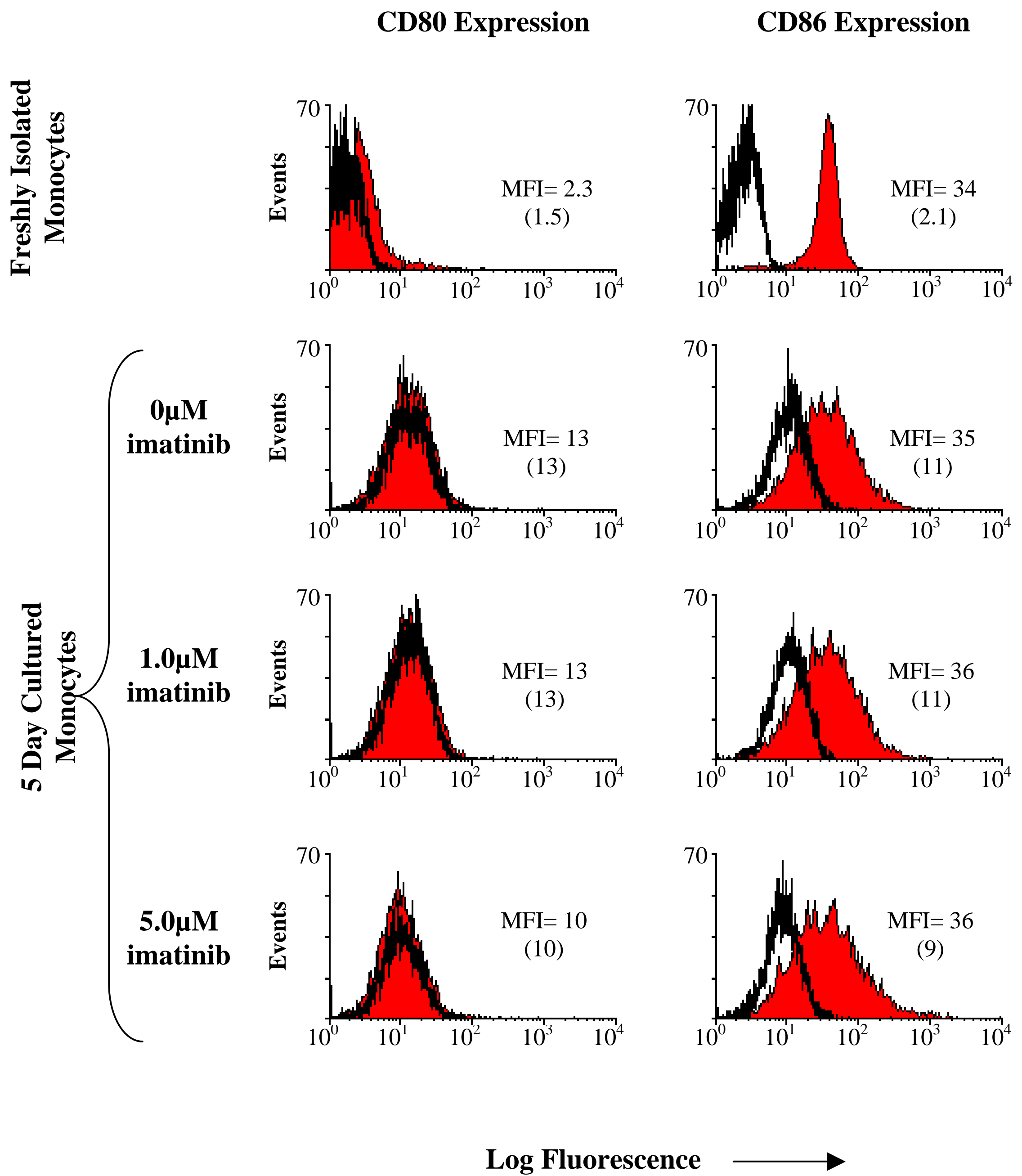
To determine whether inhibition of the MLR by M-CSF and GM-CSF stimulated CM may be explained by reduced expression of costimulatory molecules, CM were examined for CD80 and CD86 expression following culture in the presence or absence of imatinib (Figures 5.14 and 5.15). CD80 expression was not observed on freshly isolated monocytes, or on monocytes that had been cultured for 5 days in the presence of M-CSF (Figures 5.14). While CD86 expression was observed on freshly isolated monocytes, as well as on M-CSF stimulated monocytes cultured for 5 days, the addition of 1.0μM or 5.0μM imatinib had no effect on expression levels (Figure 5.14).

GM-CSF stimulated CM also failed to express CD80, but expressed higher levels of CD86 than M-CSF stimulated CM. For example, the MFI for CD86 expression in M-CSF stimulated CM was 35 units, versus 80 units in GM-CSF stimulated CM (Figures 5.14 and 5.15). Treatment of GM-CSF stimulated CM with imatinib reduced the mean fluorescence intensity for CD86 expression by 25% at 1.0μM imatinib and by 35% at 5.0μM imatinib, although this did not appear to have any effect on the stimulatory capacity of these cells since control cultures were unable to stimulate an MLR (Figure 5.15).

A higher level of fluorescence was observed for isotype controls following culture of monocytes for 5 days for both M-CSF and GM-CSF stimulated CM when compared to freshly isolated monocytes (Figure 5.14 and 5.15). This was due to an increase in autofluorescence that is characteristic of CM.

Figure 5.14 Effect of Imatinib on the Expression of CD80 and CD86 on M-CSF Stimulated Cultured Monocytes.

Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with M-CSF (40ng/mL) in the presence or absence of imatinib for a period of 5 days. Cells were then harvested and examined for expression of CD80 and CD86. CD80 expression was absent on freshly isolated monocytes, and on monocytes that had been stimulated with M-CSF. Although CD86 expression was observed on freshly isolated monocytes and M-CSF stimulated cultured monocytes, expression was not modulated by imatinib treatment. Results are representative of three individual experiments using different donors. Unfilled histograms indicate background fluorescence following staining with isotype controls. The marker tool was set based on the maximum fluorescence of the isotype control. MFI = mean fluorescence intensity, and bracketed numbers indicate the MFI value for isotype controls.



CD80 Expression
CD86 Expression
Freshly Isolated Monocytes
5 Day Cultured Monocytes
0µM imatinib
1.0µM imatinib
5.0µM imatinib
Events
70
MFI= 2.3 (1.5)
MFI= 34 (2.1)
MFI= 13 (13)
MFI= 35 (11)
MFI= 13 (13)
MFI= 36 (11)
MFI= 10 (10)
MFI= 36 (9)
10^0 10^1 10^2 10^3 10^4
Log Fluorescence

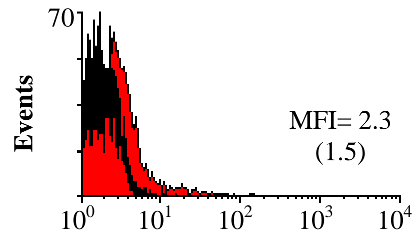
Figure 5.15 Effect of Imatinib on the Expression of CD80 and CD86 on GM-CSF Stimulated Cultured Monocytes.

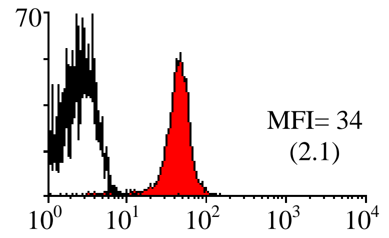
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) in the presence or absence of imatinib for a period of 5 days. Cells were then harvested and examined for expression of CD80 and CD86. CD80 expression was not observed on freshly isolated monocytes, or on monocytes that had been stimulated to differentiate following GM-CSF stimulation. CD86 expression was observed on freshly isolated monocytes and GM-CSF stimulated cultured monocytes, and expression was reduced by 25% at 1.0μM imatinib, and 35% at 5.0μM imatinib. Unfilled histograms indicate background fluorescence following staining with isotype controls. The marker tool was set based on the maximum fluorescence of the isotype control. MFI = mean fluorescence intensity, and bracketed numbers indicate the MFI value for isotype controls.

CD80 Expression

CD86 Expression

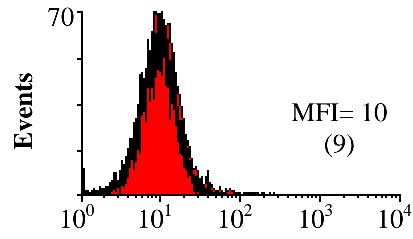
Freshly Isolated Monocytes

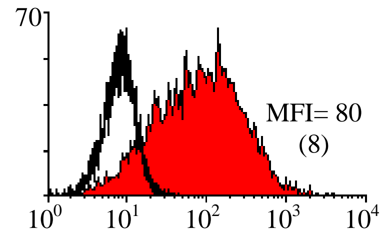




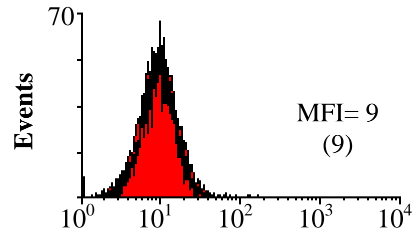
5 Day Cultured Monocytes

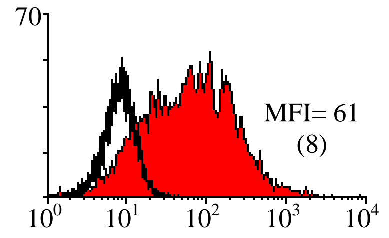
0µM imatinib



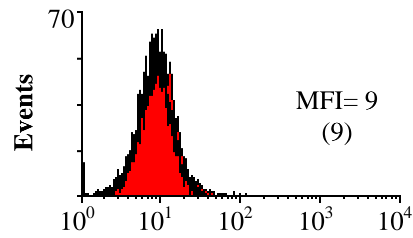


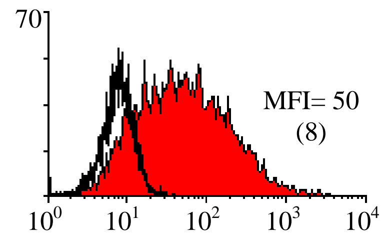
1.0µM imatinib





5.0µM imatinib





Log Fluorescence

5.3 Discussion

In the previous chapter, it was demonstrated the development of the monocyte/macrophage lineage in normal donors was inhibited by therapeutic concentrations of imatinib. To address the potential clinical significance of altered immunological function of monocytes/macrophages, the modulation of monocyte/macrophage function by imatinib was examined in this chapter.

The addition of imatinib to monocyte cultures reduced the morphological and functional response of CM to the potent activator LPS, with a decrease in the extent of pseudopodia formation, as well as a decrease in the production of proinflammatory cytokines. The magnitude of inhibition by imatinib on IL-6 and TNF-α production varied depending on the growth stimulus used, as M-CSF stimulated CM were sensitive to the drug at 0.3μM imatinib, while CM stimulated with GM-CSF or a combination of M-CSF and GM-CSF were affected at 1.0μM imatinib and 5.0μM imatinib respectively. Reduced production of IL-6 and TNF-α was not attributable to a decrease in cell number, as no loss of viability up to an imatinib concentration of 5.0μM was shown in Chapter 4. Absence of a toxic effect of imatinib on non-malignant haemopoietic cells is also supported by published data, where *in vitro* exposure of normal $CD34^+$ cells to 10.0μM imatinib for 48 hours did not induce apoptosis or lead to a significant G1-arrest (Bartolovic *et al.* 2003).

The phagocytic capacity of macrophages is pivotal to their role as antigen-presenting cells, and occurs following interactions of the foreign particle with various cell surface receptors. These interactions can be either opsonin-dependent and mediated by Fc and complement receptors, or opsonin-independent and mediated by mannose receptors (Ofek *et al.* 1995; Reichner *et al.* 2001). Experiments were performed to determine whether imatinib impaired the ability of CM to phagocytose non-opsonised and opsonised zymosan particles that consist of mannan and β-glucan.

The effect of imatinib on Fc and mannose receptor mediated phagocytosis, as well as LPS activation, was of greater magnitude and apparent at lower concentrations in M-CSF stimulated CM than GM-CSF stimulated CM. The results suggest that although GM-CSF may predominantly stimulate monocyte/macrophage development through autocrine

induction of M-CSF (Horiguchi *et al.* 1987; Gruber and Gerrard 1992), the effects of which are inhibited by imatinib, GM-CSF also provides additional cell stimulation that is independent of M-CSF.

It was also observed that mannose receptor mediated phagocytosis was more susceptible to imatinib treatment than Fc receptor mediated phagocytosis in both M-CSF and GM-CSF stimulated cultures, suggesting that expression of mannose receptors is more vulnerable to imatinib treatment than expression of Fc receptors. Possible mechanisms include preferential downregulation of mannose receptor expression in the presence of imatinib, or a failure of cultured cells to acquire expression of the mannose receptor. Alternatively, decreased phagocytosis of non-opsonised zymosan by imatinib may be attributed to an inhibition of the mannose signalling pathway by imatinib. The consequence of imatinib treatment on the expression of Fc and mannose receptors by CM may be clarified using flow cytometry, but was not examined in this study.

The ability of macrophages to detect and phagocytose antigenic material, as well as respond to LPS in bacterial cell walls, is pivotal to their role in innate immunity. Macrophages also play a crucial role in initiating adaptive immune response, by presenting processed antigen to T cells. Whether imatinib altered the ability of CM to activate T cell responses was therefore examined in this study using a mixed lymphocyte reaction (MLR). Treatment of M-CSF stimulated CM with imatinib was found to impair the ability of these cells to activate responder cells in an MLR, while GM-CSF stimulated CM were less able to activate an MLR, even in the absence of imatinib.

T cell recognition of an antigenic peptide on the surface of antigen presenting cells in the absence of adequate costimulation can induce a state of inactivation where T cells are unable to proliferate. The results presented in this chapter suggest that a reduction in MLR stimulation by M-CSF stimulated CM is not due to reduced availability of costimulatory molecules, as although CD80 expression was not observed, expression of CD86 was consistent at each dose of imatinib. Furthermore, expression of CD86 alone was insufficient to render GM-CSF stimulated CM capable of inducing an MLR. HLA-DR expression was shown to be unaffected by imatinib treatment of monocytes in Chapter 4

and it therefore also seems unlikely that failure to present alloantigen accounts for the decreased ability of imatinib-treated CM to stimulate an MLR.

An alternative mechanism of imatinib inhibition of an MLR may be through diminished response to stimulatory cytokines produced by T cells such as interferon gamma (Pace *et al.* 1983). In addition, imatinib treated M-CSF stimulated CM may be impaired in their activation of responder cells through reduced stability in the contact between activating cells and responding cells. This may be associated with decreased expression of adhesion molecules by M-CSF stimulated CM such as intracellular adhesion molecule-1 (ICAM-1). ICAM-1 interacts with other adhesion molecules on the surface of T cells such as LFA-1, providing an important costimulatory signal and mediating a low affinity adhesion between the cells (Van Seventer *et al.* 1990; Tibbetts *et al.* 1999). Decreased pseudopodia formation, as observed in Chapter 4, may also reduce cell contact and contribute to suppression of the MLR, although other factors are clearly involved as GM-CSF stimulated CM were unable to activate an MLR, even in the absence of imatinib.

Table 5.1. Concentration of imatinib at which the functional response of cultured monocytes stimulated with M-CSF or GM-CSF is significantly suppressed.

Parameter	**M-CSF Stimulated CM**	**GM-CSF Stimulated CM**
IL-6 Production Following LPS Activation	0.3μM	1.0μM
TNF-α Production Following LPS Activation	0.3μM	1.0μM
Phagocytosis of Non-Opsonised Zymosan	1.0μM	5.0μM
Phagocytosis of Opsonised Zymosan	5.0μM	5.0μM
Suppression of MLR	1.0μM	Unable to stimulate

Table 5.1 summarises the effect of imatinib on the function of monocytes stimulated with M-CSF or GM-CSF, and demonstrates the preferential inhibition of monocytes cultured in the presence of M-CSF. A significant reduction in the acquisition of functional capacity by CM was observed following treatment with imatinib and suggests that long-term

treatment of patients with imatinib has the potential to impair immunological function, particularly in response to infections requiring mononuclear phagocyte action. The *in vitro* suppression induced by imatinib on the function of cells of the monocyte/macrophage lineage further supports the hypothesis that the signalling pathway of monocyte/macrophage development may be affected. The next and final results chapter explores the signalling pathways that are involved in the inhibition of monocyte/macrophage development and function by imatinib.

Chapter 6:

MECHANISM OF IMATINIB SUPPRESSION OF MONOCYTE/MACROPHAGES

6.1 Introduction

In Chapters 4 and 5, it was demonstrated that the tyrosine kinase inhibitor imatinib mediated specific suppression on cells of the monocyte/macrophage lineage from normal donors, when stimulated with M-CSF and/or GM-CSF. In particular, the growth of monocyte/macrophage colonies from BM progenitor cells and the differentiation of cultured monocytes were significantly inhibited at 0.86μM-1.0μM imatinib, which is within the therapeutic dose range. Functional assays such as LPS activation, phagocytosis, and stimulation of T cells using cultured monocytes were also inhibited by imatinib and this was most marked following stimulation of cells with M-CSF. These results suggest that imatinib may target as yet undefined tyrosine kinase(s).

Currently identified imatinib targets include Abl, platelet derived growth factor receptors (PDGFR), ABL-related gene (ARG), and c-kit tyrosine kinases (Carroll *et al.* 1997; Buchdunger *et al.* 2000; Okuda *et al.* 2001). Abl is thought to be important in cell growth and arrest, the modulation of apoptosis and DNA repair (reviewed in Shaul 2000), and while mice expressing mutated Abl have decreased neonatal viability and lymphopenia (Tybulewicz *et al.* 1991), there is no direct evidence of a role for Abl in haemopoietic development. ARG is widely expressed and plays an important role in the central nervous system but, similarly to Abl, it is not involved in normal haemopoiesis (Okuda *et al.* 2001). It is therefore unlikely that blockade of Abl or ARG by imatinib accounts for the inhibition observed in Chapters 4 and 5 and this is further supported by the observation that the IC_{50} for Abl and ARG is approximately 10-100 fold lower than the IC_{50} observed for monocyte/macrophage growth (Druker *et al.* 1996; Okuda *et al.* 2001).

SCF plays an important role in haemopoiesis, and its receptor c-kit is expressed on a range of haemopoietic cells including mast cells, megakaryocytes and $CD34^+$ progenitor cells, as well as on non-haemopoietic cells (Ashman 1999). SCF does not support significant colony formation alone, although it acts synergistically with other cytokines such as EPO, IL-3, GM-CSF and G-CSF to increase the growth of macrophage, megakaryocytic and erythroid colonies (McNiece *et al.* 1991). Since Chapter 4 demonstrated that inhibition of monocyte/macrophage growth by imatinib was observed in the absence of added SCF, it is unlikely that an inhibition of c-kit signalling was responsible for the suppressive effect

observed in this study. The contribution of endogenously produced SCF and/or an upregulation of c-kit expression during culture, however, cannot be ruled out.

Inhibition of PDGFRα and PDGFRβ kinase activity by imatinib has also been demonstrated at IC_{50} values of 0.1μM (Buchdunger *et al.* 2000). PDGF is produced by a range of cell types that include platelets, fibroblasts, keratinocytes, neurons and macrophages, and this pleiotropic cytokine is involved in the proliferation of mesenchymal cells, physiological repair of wounds and the pathogenesis of various proliferative diseases (reviewed in Heldin and Westermark 1999; Rosenkranz and Kazlauskas 1999). Within the haemopoietic system, PDGF stimulates multilineage haemopoietic precursor cells and long-term culture-initiating cells, as well as erythropoiesis (Dainiak *et al.* 1983; Yan *et al.* 1993; Su *et al.* 2002). With regard to $CD34^+$ cells, the mechanism by which PDGF enhances cell growth is unknown, and may be attributed to secondary effects on accessory cells rather than acting directly on $CD34^+$ cells themselves (Su *et al.* 2002). A secondary effect is supported by the observation that PDGF receptors are not detected on freshly isolated $CD34^+$ cells, with expression requiring 2-3 days of culture in the presence of cytokines (Su *et al.* 2002). This is further supported by the observation that while PDGF stimulates murine colony-forming unit-granulocyte-monocyte (CFU-GM) proliferation from bone marrow cells, PDGF has no effect on CFU-GM proliferation by $CD34^+$ cells (Yang *et al.* 2001).

Although expression of PDGFRβ has been demonstrated on monocyte-derived macrophages (Inaba *et al.* 1993), it is unlikely that inhibition of PDGF receptors by imatinib can account for the inhibitory effect on monocyte/macrophage development and function observed in Chapters 4 and 5. Colony assays were established in the presence of serum, which is known to contain PDGF (Childs *et al.* 1982), however, colony growth was initiated by $CD34^+$ cells which are not known to express PDGF receptors.

The known imatinib targets therefore do not appear to account for the observed inhibitory effect of imatinib on monocyte/macrophage development and function. A likely mechanism is inhibition of a signalling pathway involved in monocyte/macrophage development, although in contrast to the related class III receptor tyrosine kinases c-kit,

PDGFRα and PDGFRβ, phosphorylation of the M-CSF receptor, c-fms is reportedly unaffected by imatinib up to a concentration of 10.0μM (Buchdunger *et al.* 2000). The receptor c-fms is crucial for the growth and differentiation of the monocyte-macrophage lineage (Sherr 1990) and upon ligand binding to the extracellular domain of c-fms, the receptor is induced to dimerize and trans-autophosphorylate several cytoplasmic tyrosine residues (Bourette and Rohrschneider 2000). The phosphorylated cytoplasmic domain subsequently interacts with primary adapter proteins, which then initiate signalling along specific pathways to effect the differentiation and functional maturation of monocytes.

Inhibition of c-fms signalling by imatinib may account for the inhibitory effect observed in Chapters 4 and 5, following stimulation of cells with M-CSF. GM-CSF stimulation of monocyte development was also inhibited by imatinib, albeit at a higher concentration, and this effect may also be attributable to an inhibition of c-fms signalling since GM-CSF stimulation of human monocytes induces autocrine production of M-CSF (Horiguchi *et al.* 1987; Gruber and Gerrard 1992). This chapter therefore examines the hypothesis that c-fms is inhibited by imatinib at therapeutically relevant concentrations.

6.2 Results

6.2.1 Effect of anti-c-kit on Haemopoietic Colony Formation

In order to exclude a possible role for c-kit in imatinib mediated suppression of monocyte/macrophage development, preliminary experiments were performed to examine whether endogenous production of SCF was contributing to colony growth in 4HGF stimulated cultures. Colony cultures were established using bone marrow mononuclear cells from normal donors, and stimulated with 4HGF and 5HGF in the presence or absence of 0.3µM imatinib. An antibody that blocked c-kit was also added to cultures at a concentration of 1.0µg/mL, to examine the effect of this antibody on imatinib-mediated inhibition of colony growth.

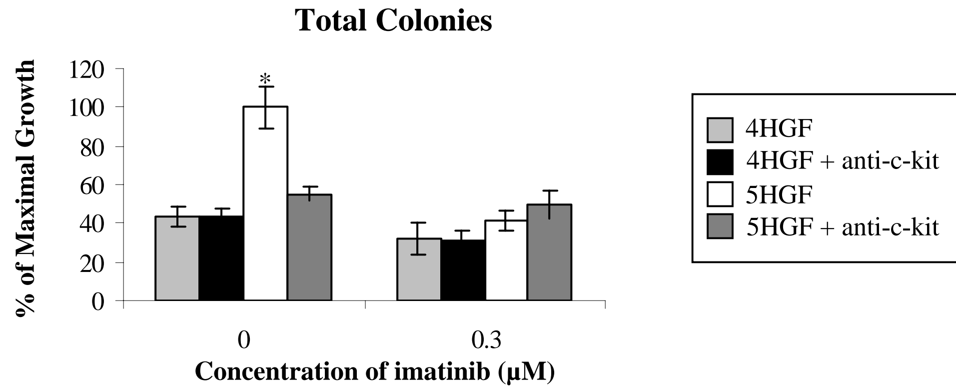
The dose of anti-c-kit used in this experiment was sufficient to completely block the SCF receptor, as addition of anti-c-kit to cultures stimulated with 4HGF plus SCF (5HGF) reduced total colony growth to the same level as cultures stimulated with 4HGF alone (Figure 6.1A). In the absence of imatinib, the addition of anti-c-kit to cultures stimulated with 4HGF had no effect on total colony number, demonstrating that endogenous production of SCF was not occurring (Figure 6.1A).

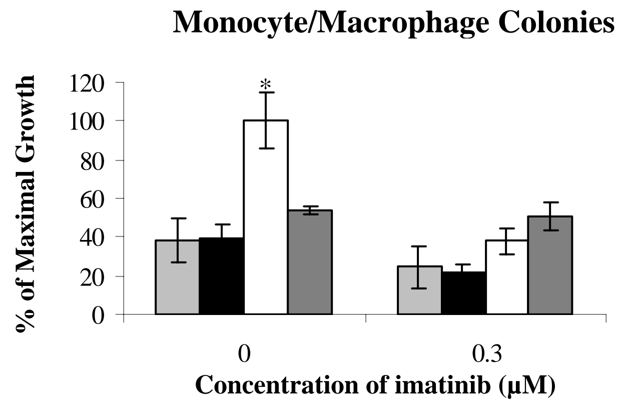
In accordance with results from Chapter 4, the addition of 0.3µM imatinib to cultures stimulated with 5HGF reduced total colony growth to the level observed with 4HGF (Figure 6.1A). The reduction in total colony growth at 0.3µM imatinib in 5HGF stimulated cultures was equivalent to 5HGF stimulated cultures that were treated with anti-c-kit antibodies, further indicating that 0.3µM imatinib was sufficient to completely block the SCF receptor (Figure 6.1A).

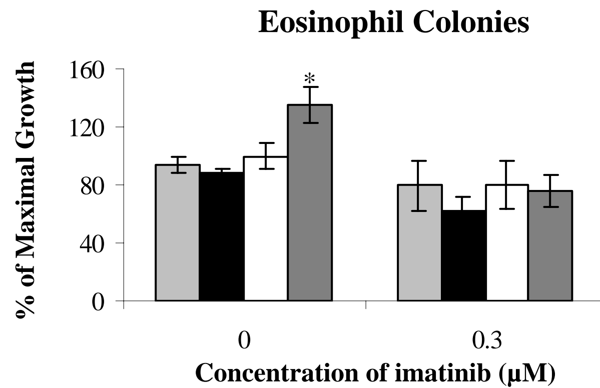
The effect of abrogating c-kit signalling was also examined on monocyte/macrophage (Figure 6.1B), eosinophil (Figure 6.1C) and neutrophil (Figure 6.1D) colony formation. The addition of 1.0µg/mL anti-c-kit to 4HGF stimulated cultures had no effect on monocyte/macrophage, eosinophil or neutrophil growth. Furthermore, the concentration of anti-c-kit was sufficient to completely abrogate the growth enhancing effect of added SCF for each of the colony types (Figures 6.1B-6.1D).

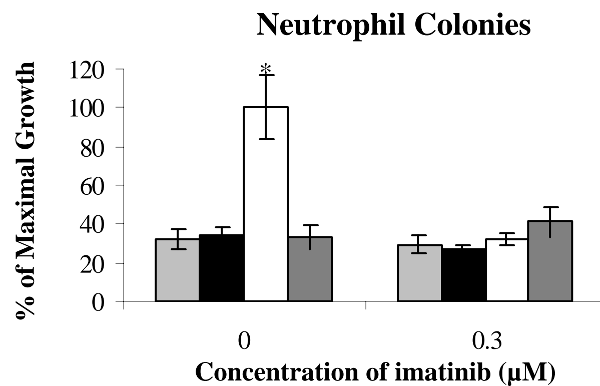
Figure 6.1 Effect of Anti-c-kit and Imatinib on the Growth of Colonies Stimulated with 4HGF or 5HGF.

Mononuclear cells were isolated from the bone marrow of normal donors and grown in semi-solid agar supplemented with 4HGF (IL-3, IL-6, G-CSF, GM-CSF (each at a final concentration of 10ng/mL)), 4HGF plus anti-c-kit antibodies, 5HGF (IL-3, IL-6, G-CSF, GM-CSF, SCF (each at a final concentration of 10ng/mL)), or 5HGF plus anti-c-kit antibodies, for a period of 14 days. The addition of anti-c-kit antibodies (1μg/mL) to 4HGF cultures had no effect on total colony growth (**A**), monocyte/macrophage colony growth (**B**), eosinophil colony growth (**C**), or neutrophil colony growth (**D**). The concentration of anti-c-kit antibody used was sufficient to completely block the receptor, as the addition of anti-c-kit to 5HGF stimulated colonies reduced the level of growth to that of 4HGF stimulated cultures. The addition of 0.3μM imatinib reduced colony growth in 5HGF stimulated cultures to the level observed in 5HGF plus anti-c-kit cultures or 4HGF cultures, indicating that 0.3μM imatinib was sufficient to completely block c-kit. The 4HGF control was used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments, each performed in triplicate, using different donors. Statistical significance was determined relative to the 4HGF control (* denotes $p < 0.05$).


A
Total Colonies
% of Maximal Growth
120
100
80
60
40
20
0
*
0
0.3
Concentration of imatinib (μM)
4HGF
4HGF + anti-c-kit
5HGF
5HGF + anti-c-kit


B
Monocyte/Macrophage Colonies
% of Maximal Growth
120
100
80
60
40
20
0
*
0
0.3
Concentration of imatinib (μM)


C
Eosinophil Colonies
% of Maximal Growth
160
120
80
40
0
*
0
0.3
Concentration of imatinib (μM)


D
Neutrophil Colonies
% of Maximal Growth
120
100
80
60
40
20
0
*
0
0.3
Concentration of imatinib (μM)

6.2.2 Effect of anti-c-fms on Monocyte/Macrophage Colony Formation

In Chapter 4, it was demonstrated that imatinib inhibited the growth of monocyte/macrophage colonies, suggesting that a specific inhibition of monocyte/macrophage signalling pathways was occurring. The role of c-fms in colony growth was therefore examined using an anti-c-fms antibody (Figure 6.2).

In M-CSF stimulated cultures, only monocyte/macrophage colony growth was observed, and this growth was inhibited by approximately 80% at 1.0μM imatinib (Figure 6.2A). The addition of an anti-c-fms antibody at 1.0μg/mL was sufficient to completely inhibit monocyte/macrophage growth (Figure 6.2A).

As observed in Chapter 4, stimulation of colony formation with GM-CSF induced growth of both monocyte/macrophage and eosinophil colonies (Figure 6.2B). The addition of 1.0μM imatinib reduced monocyte/macrophage growth by approximately 80% and eosinophil growth was unaffected by imatinib until 10.0μM, where growth was 40% lower than controls (Figure 6.2B). Interestingly, the addition of an anti-c-fms antibody to GM-CSF stimulated cultures completely abrogated monocyte/macrophage colony growth, but had no effect on eosinophil growth (Figure 6.2B).

6.2.3 Analysis of Autocrine M-CSFProduction

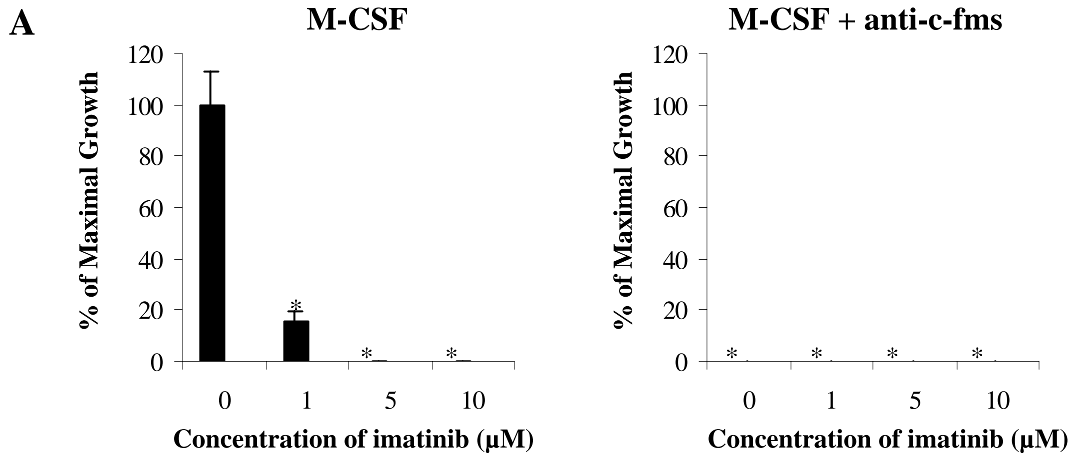
Since GM-CSF appeared to indirectly stimulate monocyte/macrophage colony formation by signalling through c-fms, the hypothesis that GM-CSF induced autocrine production of M-CSF was tested. To address this, monocyte cultures were established from the peripheral blood of normal donors, and stimulated with GM-CSF for a period of 5 days in the presence or absence of imatinib. Supernatants were harvested at 24 hour time points, and assayed for M-CSF by ELISA.

A standard curve was generated to enable calculation of M-CSF levels in the monocyte supernatants (Figure 6.3A), and low levels (20pg/mL) of M-CSF were detected 24 hours after cultures were established (Figure 6.3B). Levels of M-CSF increased steadily over the 5 days in culture and approximately 70pg/mL of M-CSF was detected in day 5 supernatants collected from cultures not treated with imatinib. The addition of 1.0μM

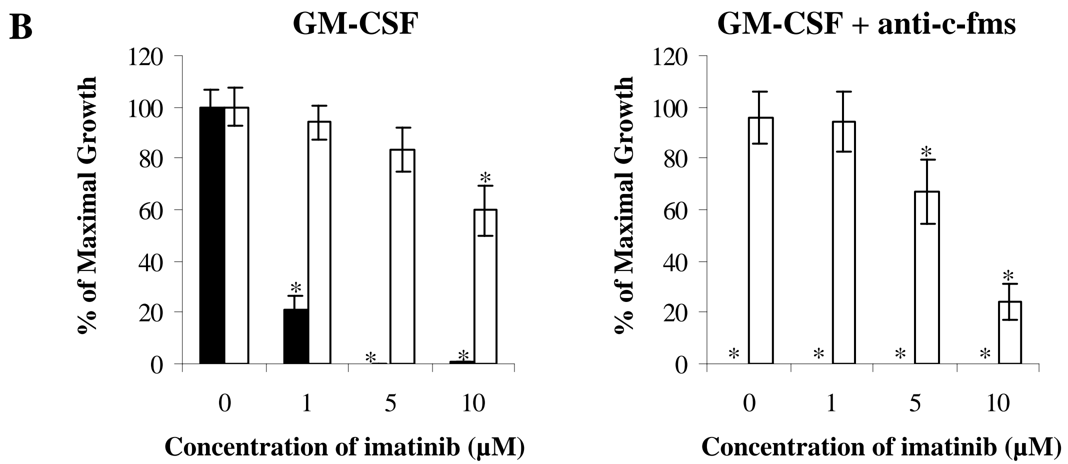
Figure 6.2 Effect of Anti-c-fms and Imatinib on the Growth of Colonies Stimulated with M-CSF or GM-CSF.

$CD34^+$ cells were isolated from the bone marrow of normal donors and grown in semi-solid agar supplemented with M-CSF (25ng/mL) (**A**) or GM-CSF (10ng/mL) (**B)** for a period of 14 days. The effect of anti-c-fms antibodies (1µg/mL) and imatinib on colony growth was examined, and the addition of 1.0µM imatinib reduced monocyte/macrophage colony growth by 80% following stimulation with either M-CSF or GM-CSF. Eosinophil growth was only observed following GM-CSF stimulation, and was reduced by approximately 40% at 10.0µM imatinib. Monocyte/macrophage growth was abrogated in the presence of α-c-fms antibodies following stimulation with either M-CSF or GM-CSF, while eosinophil growth was unaffected until 10.0µM imatinib. The 0µM imatinib control was used as the reference value for maximal growth, and all other data points were normalised to this value. Results represent the mean (±SEM) of normalised data from 2 individual experiments, each performed in triplicate, using different donors. Statistical significance was determined relative to the 0µM imatinib control (* denotes $p < 0.05$).

A



B



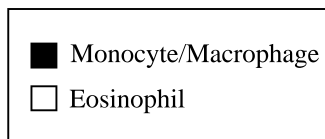
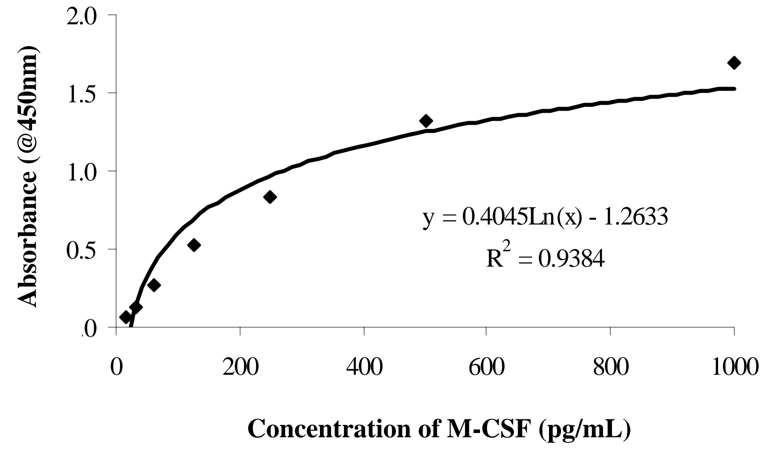


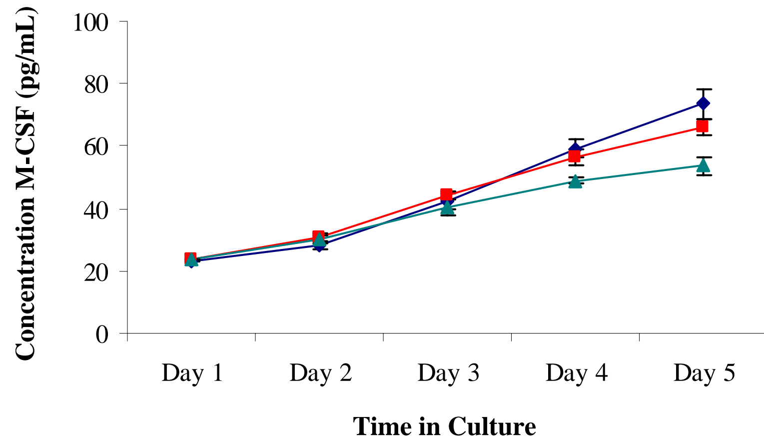
Figure 6.3 Analysis of M-CSF Production by GM-CSF Stimulated Monocyte Cultures.

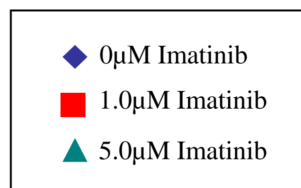
Peripheral blood monocytes were isolated from normal donors and grown in serum deprived medium supplemented with GM-CSF (20ng/mL) for five days. Supernatants were harvested at 24 hour time points, and assayed for M-CSF production by ELISA. A standard curve was established using known standards (**A**), and used to calculate the concentration of M-CSF in culture supernatants (**B**). In control cultures, approximately 20pg/mL M-CSF was produced within 24 hours of the initiation of the culture, and this increased to approximately 70pg/mL after 5 days. A concentration of 1.0μM imatinib had no effect on M-CSF production by monocyte cultures, while a slight reduction was observed in the presence of 5.0μM imatinib on day 5. Results are representative of data obtained from three individual experiments using different donors.

A



B





imatinib had no effect on M-CSF production by the cultured monocytes, while a 30% decrease in M-CSF production at day 5 was seen at 5.0μM imatinib.

6.2.4 Effect of Imatinib on an M-CSF-Dependent Cell Line

To further examine the role of c-fms in imatinib inhibition of monocyte/macrophage development and function, the effect of imatinib on a cell line that was dependent on human M-CSF was investigated. The murine myeloid cell line FDC-P1 is dependent on murine IL-3 or murine GM-CSF for growth, and undergoes division once every 12 hours. This cell line was infected by virus particles containing human c-fms, and transformed cells were selected by growth in medium supplemented with rhM-CSF. Expression of c-fms was confirmed using flow cytometry. These cells were termed "FDC-cfms", and were dependent on murine IL-3, murine GM-CSF or rhM-CSF for growth.

6.2.4.1 Effect of Imatinib on the Proliferation of FDC-cfms Cells

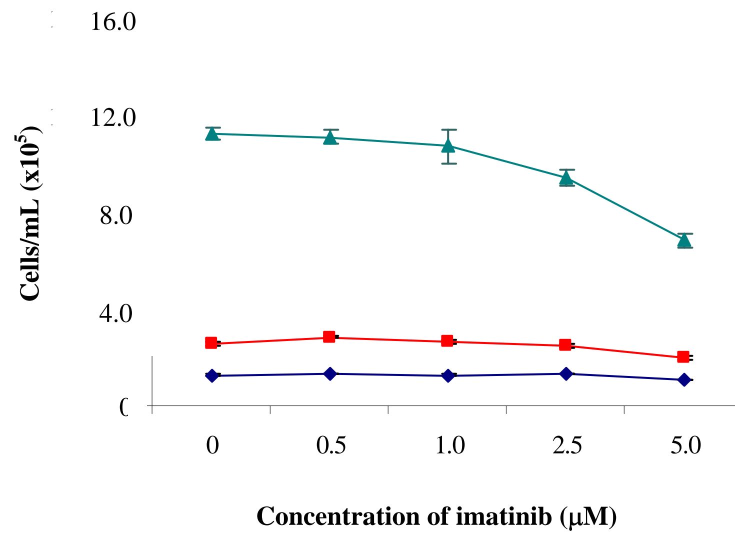
To examine whether imatinib altered the growth kinetics of M-CSF stimulated FDC-cfms cells, cultures were established at 5.0×10^4 cells/mL and stimulated with either murine IL-3 or rhM-CSF for 48 hours. Cell counts were performed at 12, 24 and 48 hours, and in control cultures stimulated with IL-3 alone, no imatinib-specific effect on growth was observed at 12 or 24 hours across the range of imatinib doses examined (Figure 6.4A). At 48 hours, FDC-cfms proliferation in the presence of IL-3 was not affected by 0.5μM or 1.0μM imatinib, however at 2.5μM imatinib, cell counts were reduced by 15%. At 5.0μM imatinib, cell counts were 40% lower than controls (Figure 6.4A).

Where M-CSF was the sole source of stimulation, the cell count was 6.8×10^4 cells/mL at 5.0μM imatinib 12 hours after the initiation of the culture, indicating that only approximately 18% of starting cells underwent cell division (Figure 6.4B). At 24 hours, cell counts were approximately 45% lower at 2.5μM imatinib than cultures not treated with imatinib, and at 5.0μM imatinib, cell counts were lower than the seeded level (Figure 6.4B). The effect of imatinib on M-CSF stimulated FDC-cfms cultures was most profound at 48 hours, where 2.5μM imatinib reduced cell counts by approximately 80% relative to controls (Figure 6.4B). The cell count at 2.5μM imatinib was only slightly higher at 48 hours than at 12 or 24 hours, further indicating that these cells were impaired in their

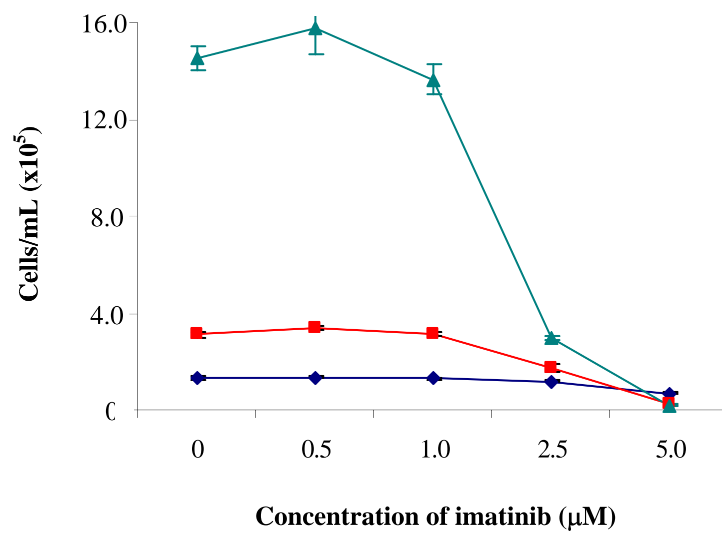
Figure 6.4 Effect of Imatinib on the Growth of FDC-c-fms Cells Stimulated with Murine IL-3 or Human M-CSF.

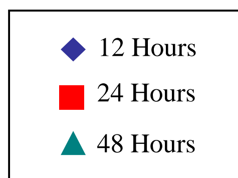
The murine cell line FDC-P1 was infected with the human monocyte colony stimulating factor receptor, c-fms ("FDC-cfms"), and was dependent on mIL-3, mGM-CSF or rhM-CSF for growth. The effect of imatinib on mIL-3 (1/2000) (**A**) or rhM-CSF (60ng/mL) (**B**) stimulated proliferation was examined by performing cell counts at 12, 24 and 48 hours, with starting cultures initiated at $5.0x10^4$/mL. In control cultures stimulated with mIL-3, no imatinib-specific effect on cell growth was observed at 12 or 24 hours across the range of imatinib doses examined (**A**). At 48 hours, cell counts were reduced by 15% at 2.5μM imatinib, and by 40% at 5.0μM imatinib. In cultures stimulated with rhM-CSF alone, the cell count was similar to the seeded value at 5.0μM imatinib, 12 hours after the initiation of the culture. At 24 hours, cell counts were approximately 45% lower at 2.5μM imatinib compared to control cultures (**B**). The effect of imatinib on M-CSF stimulated FDC-cfms cultures was most marked at 48 hours, where 2.5μM imatinib reduced cell counts by approximately 80% relative to controls. Data points represent the mean (±SEM) of normalised results, relative to the 0μM imatinib control, from one experiment established in triplicate. Results are representative of three individual experiments using two individually infected cell lines.

A



B





ability to undergo cell division. At 48 hours, the concentration of cells at 5.0μM imatinib remained lower than the seeded level (Figure 6.4B).

6.2.4.1.1 Calculation of the IC_{50} for Imatinib Inhibition of FDC-cfms Proliferation

The relationship between imatinib concentration and FDC-cfms cell growth at 48 hour time points was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of growth (Figure 6.5). Treatment of IL-3 stimulated FDC-cfms cultures with imatinib across the concentration range tested did not inhibit proliferation by 50%, however the equation for the line of best fit predicted an IC_{50} value of 6.54μM imatinib (Figure 6.5A). In contrast, treatment of M-CSF stimulated FDC-cfms cultures with imatinib gave a lower IC_{50} value of 1.86μM imatinib (Figure 6.5B).

6.2.4.2 Effect of Imatinib on the Phosphorylation & Expression of c-fms

To determine if imatinib inhibited c-fms directly, the effect of imatinib on the phosphorylation of c-fms was examined on FDC-cfms cell lines (Figure 6.6). In these experiments, FDC-cfms cells were grown for 48 hours in IL-3, and then starved for 1 hour to prevent receptor phosphorylation and promote maximal expression of c-fms. Starved FDC-cfms cells were then stimulated with rhM-CSF, with preliminary experiments establishing that a stimulation time of 2 minutes was optimal for peak phosphorylation in the absence of receptor degradation. Immunoprecipitation was then performed using an anti-c-fms antibody. Prior to gel loading, a Micro BCA™ protein assay was performed to ensure equal amounts of protein were present in each immunoprecipitate. Western blots were probed using either anti-phosphotyrosine (Figure 6.6) or anti-c-fms antibodies (Figure 6.7), with experiments were performed a minimum of 3 times. The inclusion of protein standards enabled comparison of results across each individual experiment.

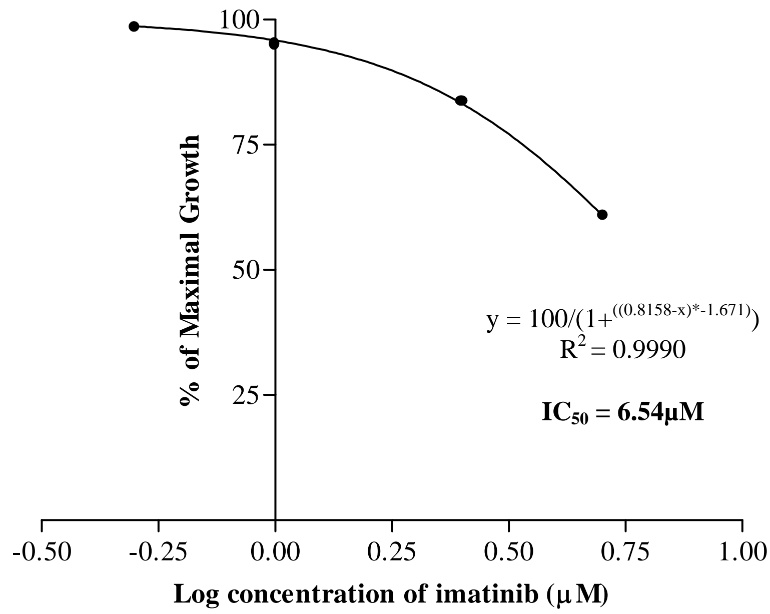
6.2.4.2.1 Phosphorylation of c-fms

Starved FDC-cfms cells that were not stimulated with M-CSF displayed no c-fms phosphorylation, and phosphorylation was not observed in lanes where M-CSF stimulated lysates were immunoprecipitated with an isotype control antibody (Figure 6.6A). Normalised data from 3 experiments demonstrated that starved FDC-cfms cells that were

Figure 6.5 Effect of Imatinib on the Growth of FDC-c-fms Cells Stimulated with Murine IL-3 or Human M-CSF.

The murine cell line FDC-P1 was infected with the human monocyte colony stimulating factor receptor, c-fms (FDC-cfms), and was dependent on mIL-3, mGM-CSF or rhM-CSF for growth. The effect of imatinib on mIL-3 (1/2000) (**A**) or rhM-CSF (60ng/mL) (**B**) stimulated proliferation was examined by performing cell counts at 12, 24 and 48 hours, and the relationship between imatinib concentration and cell growth predicted according to a sigmoidal model. The equation for the line of best fit was used to calculate the IC_{50} value for imatinib-specific inhibition of growth. The IC_{50} was calculated to be 6.54μM imatinib for IL-3 stimulated cultures, and 1.86μM imatinib for M-CSF stimulated cultures. Data points represent normalised results, relative to the 0μM imatinib control, from one experiment established in triplicate. Results are representative of three individual experiments using two individually infected cell lines.

A



B

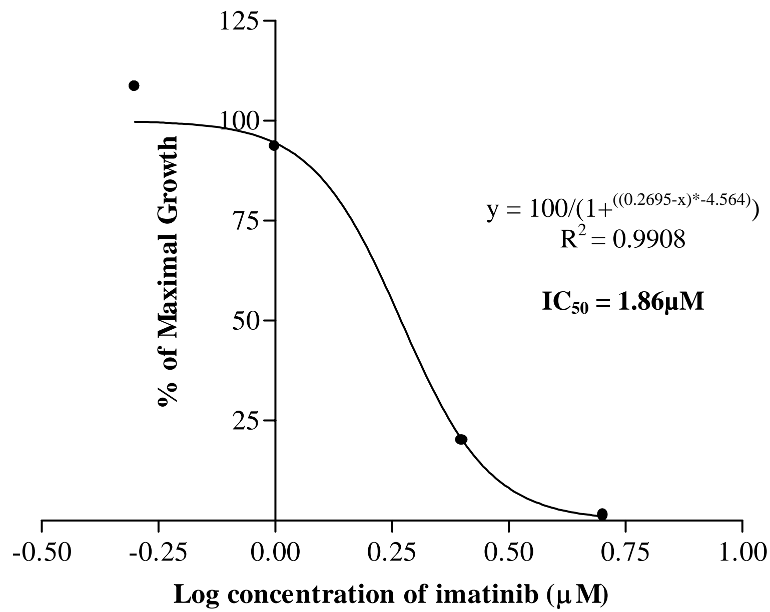
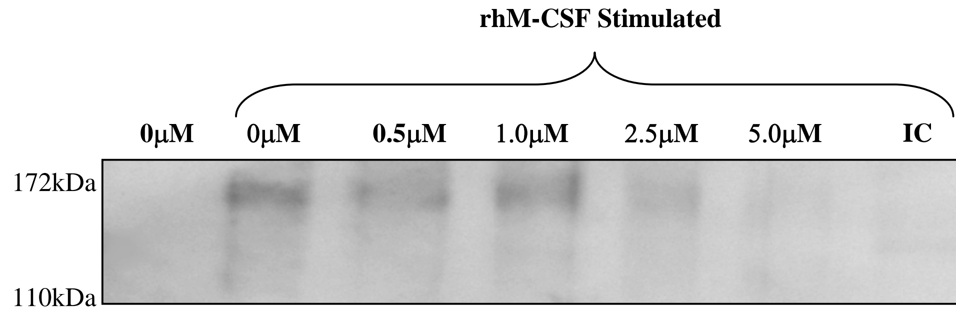
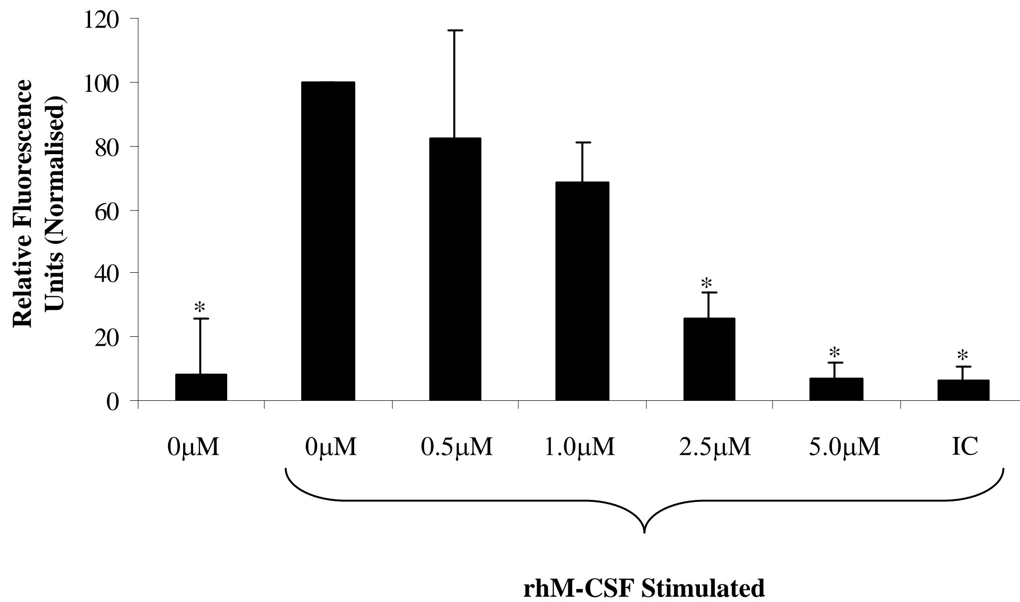


Figure 6.6 Effect of Imatinib on the Phosphorylation of c-fms Following Stimulation with Human M-CSF.

The murine cell line FDC-P1 was infected with the human monocyte colony stimulating factor receptor, c-fms (FDC-cfms), and was dependent on mIL-3, mGM-CSF or rhM-CSF for growth. Cells were starved in serum free medium for 1 hour in the presence or absence of imatinib, and then stimulated with rhM-CSF (60ng/mL) for 2 minutes. Immunoprecipitates were examined for anti-phosphotyrosine using Western blotting (IC = isotype control) (**A**). Analysis of band intensity, normalised from three Western blots, demonstrated a significant inhibition of c-fms phosphorylation by imatinib at concentrations of 2.5μM or greater (**B**). The relationship between imatinib concentration and phosphorylation was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of phosphorylation (1.42μM) (**C**). Result A is representative of three individual experiments using two individually infected cell lines, while results B and C represent the mean ($\pm$SEM) of normalised data from three individual experiments. Statistical significance was determined relative to the 0μM imatinib, M-CSF stimulated control (* denotes $p < 0.05$).


A
rhM-CSF Stimulated
0μM
0μM
0.5μM
1.0μM
2.5μM
5.0μM
IC
172kDa
110kDa


B
Relative Fluorescence Units (Normalised)
120
100
80
60
40
20
0
0μM
0μM
0.5μM
1.0μM
2.5μM
5.0μM
IC
rhM-CSF Stimulated

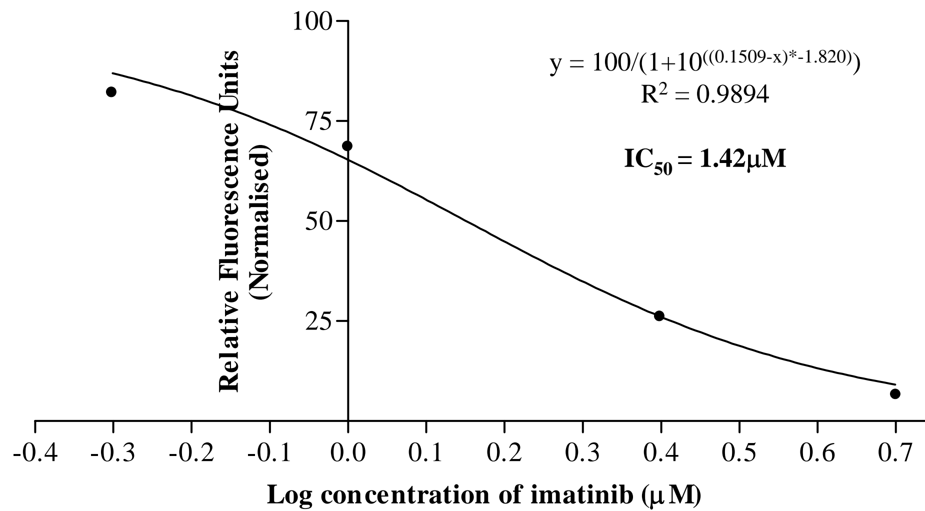
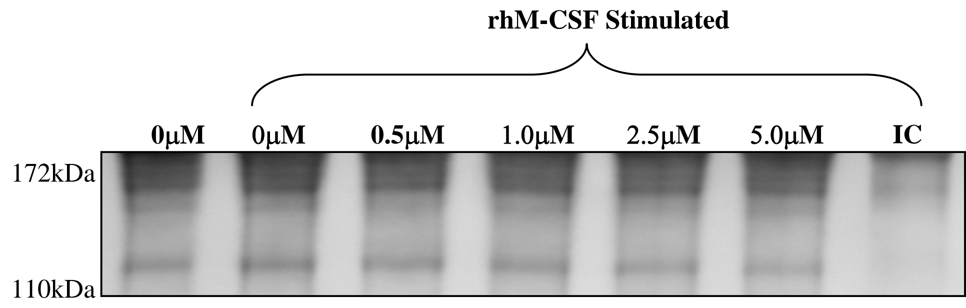
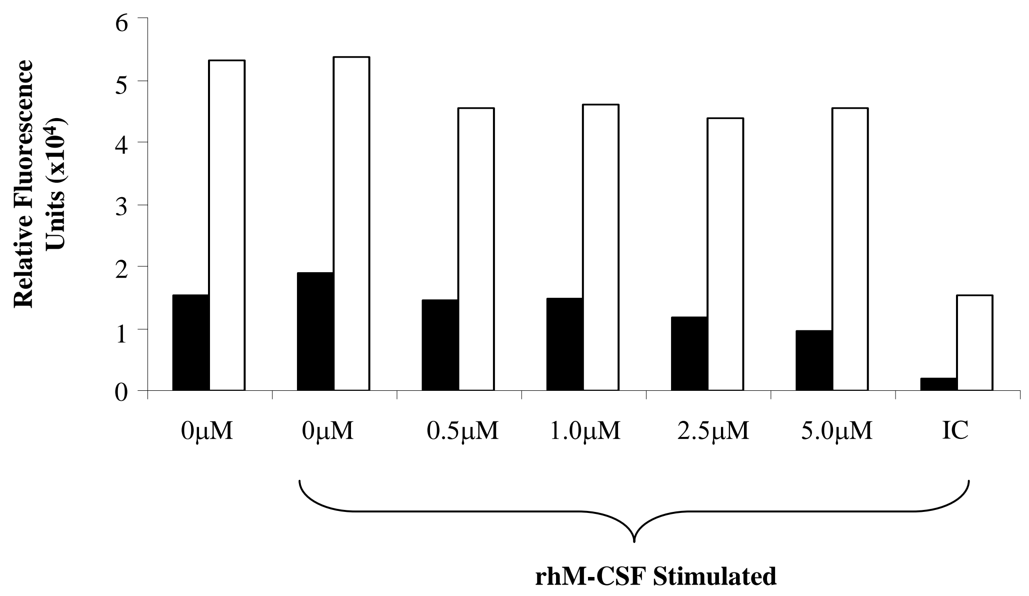

C
Relative Fluorescence Units (Normalised)
y = 100/(1+10((0.1509-x)*-1.820))
R2 = 0.9894
IC50 = 1.42μM
100
75
50
25
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Log concentration of imatinib (μM)

Figure 6.7 Effect of Imatinib on the Expression of c-fms Protein Following Stimulation with Human M-CSF.

The murine cell line FDC-P1 was infected with the human monocyte colony stimulating factor receptor, c-fms (FDC-cfms), and was dependent on mIL-3, mGM-CSF or rhM-CSF for growth. Cells were starved in serum free medium for 1 hour in the presence or absence of imatinib, and then stimulated with rhM-CSF (60ng/mL) for 2 minutes. Immunoprecipitates were examined for c-fms protein expression by Western blotting (IC = isotype control), with the 170kDa band representing the fully glycosylated c-fms protein, and the 130kDa band representing the immature, non-glycosylated form. Graphical representation of the data demonstrated that expression of the 170kDa (□) or 130kDa (■) proteins was not affected by treatment of the cells with imatinib. Results are representative of three individual experiments using two individually infected cell lines.


rhM-CSF Stimulated
0μM
0μM
0.5μM
1.0μM
2.5μM
5.0μM
IC
172kDa
110kDa


Relative Fluorescence Units (x10^4)
0
1
2
3
4
5
6
0μM
0μM
0.5μM
1.0μM
2.5μM
5.0μM
IC
rhM-CSF Stimulated

stimulated with M-CSF exhibited receptor phosphorylation, and starvation of FDC-cfms cells for 1 hour in the presence of 1.0μM imatinib reduced M-CSF mediated phosphorylation by approximately 30% (Figure 6.6B). At 2.5μM imatinib, a 75% reduction in c-fms phosphorylation was observed and at 5.0μM imatinib, no c-fms phosphorylation occurred (Figures 6.6A and 6.6B).

The relationship between imatinib concentration and phosphorylation was predicted according to a sigmoidal model, and used to calculate the IC_{50} value for imatinib-specific inhibition of phosphorylation (Figure 6.6C). An imatinib concentration of 1.42μM was required to reduce phosphorylation of c-fms by 50%, similar to the IC_{50} value for inhibition of M-CSF stimulated cell growth (Figure 6.5B).

6.2.4.2.2 Expression of c-fms: Western Blot Analysis

Since phosphorylation of the M-CSF receptor was inhibited by treatment with imatinib, Western blots were also probed for c-fms expression to confirm that this effect was not due to a decrease in c-fms expression following imatinib treatment (Figure 6.7). Two c-fms bands were detected in these blots, with the 170kDa band being the fully glycosylated c-fms protein and the 130kDa band being the immature, non-glycosylated form. The intensity of both the 170kDa and 130kDa bands was quantitated, and while much higher levels of the 170kDa protein were detected, the expression of both forms of c-fms were unaffected by imatinib treatment across the concentration range tested (Figure 6.7).

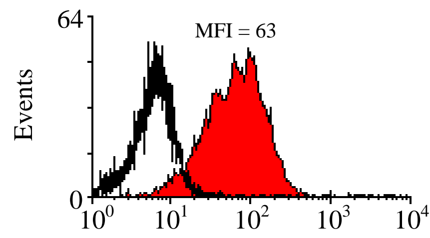
6.2.4.2.3 Expression of c-fms: Flow Cytometric Analysis

To further confirm that surface expression of c-fms was not affected by imatinib treatment, c-fms expression on FDC-cfms cells was also examined using flow cytometry (Figure 6.8). No marked differences in expression levels were observed at 0.5μM, 1.0μM or 2.5μM imatinib. At 5.0μM imatinib, c-fms expression was 40% lower than controls.

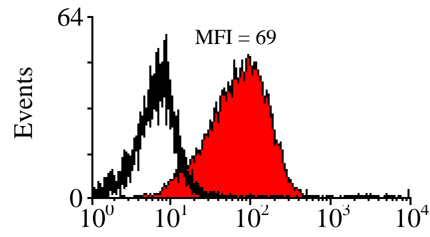
Figure 6.8 Effect of Imatinib on the Expression of c-fms Protein Following Stimulation with Human M-CSF.

The murine cell line FDC-P1 was infected with the human monocyte colony stimulating factor receptor, c-fms (FDC-cfms), and was dependent on mIL-3, mGM-CSF or rhM-CSF for growth. Cells were starved in serum free medium for 1 hour in the presence or absence of imatinib, stimulated with rhM-CSF (60ng/mL) for 2 minutes, and examined for c-fms expression by flow cytometry. Imatinib did not affect the expression of c-fms at concentrations of imatinib less than or equal to 2.5μM. A 40% reduction in expression was observed at 5.0μM imatinib. Results are representative of three individual experiments using two individually infected cell lines. MFI = mean fluorescence intensity. Unfilled histograms indicate background fluorescence following staining with isotype controls.

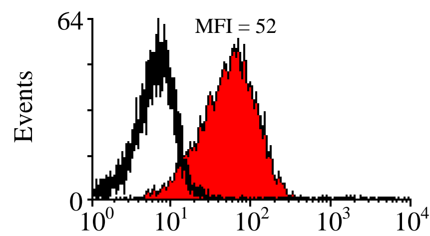
0μM imatinib



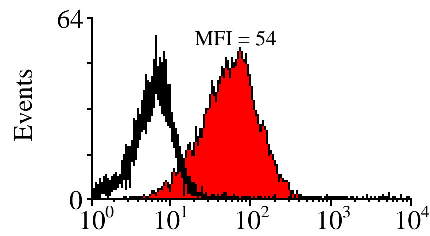
0.5μM imatinib



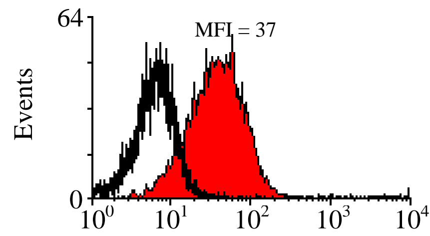
1.0μM imatinib



2.5μM imatinib



5.0μM imatinib



Log c-fms fluorescence →

6.3 Discussion

This chapter demonstrates that the known targets for the protein-tyrosine kinase inhibitor imatinib can be extended to include the M-CSF receptor, c-fms. Although the potency of inhibition is lower than what is observed for Abl (IC_{50} = 0.25μM), c-kit (IC_{50} = 0.1μM) or PDGF (IC_{50} = 0.25μM) receptor tyrosine kinases, as calculated by Western blotting (Druker *et al.* 1996; Buchdunger *et al.* 2000), inhibition was observed at concentrations of imatinib that are within the therapeutic dose range (IC_{50} = 1.42μM). This data contradicts published findings reporting that imatinib does not affect tyrosine phosphorylation of c-fms expressing NIH 3T3 cells at concentrations up to 10.0μM (Buchdunger *et al.* 2000). The analysis performed by Buchdunger *et al* (2000), however, involved Western blot analysis on whole cell lysates, and it was not stated whether specific stimulation of c-fms was performed. In this chapter, the effect of imatinib on the phosphorylation of c-fms was examined on c-fms immunoprecipitates following specific receptor stimulation with saturating doses of M-CSF. This data was further supported through demonstration that imatinib inhibited the proliferation of an M-CSF-dependent cell line at an IC_{50} of 1.86μM.

The receptor c-fms is expressed at low levels on monocytes, and its expression markedly increases during differentiation to macrophages. In the absence of M-CSF, the mature cell-surface form of c-fms is relatively stable and ligand binding downregulates receptor expression by internalisation and degradation within lysosomes (Rettenmier *et al.* 1987). Since phosphorylation of c-fms was inhibited by treatment with imatinib, Western blots were also probed for total c-fms protein to confirm this effect was not due to decreased c-fms expression. No difference in the expression of c-fms protein in the presence of imatinib was observed using either Western blotting or flow cytometry, indicating that decreased c-fms phosphorylation by imatinib was not attributable to a decrease in c-fms protein expression.

Abrogation of c-fms tyrosine phosphorylation by imatinib explains the inhibitory effect of imatinib on monocyte/macrophage growth in M-CSF stimulated colony cultures. This inhibition cannot be attributed to imatinib-blockade of c-kit tyrosine kinases in the presence of exogenous SCF. Inhibition of c-fms by imatinib is further supported by consistency in IC_{50} values in monocyte/macrophage growth and function (0.86-1.25μM

imatinib) and c-fms phosphorylation (1.47μM imatinib). Interestingly, the addition of anti-c-fms antibodies to GM-CSF stimulated colony cultures also inhibited monocyte/macrophage growth, while neither imatinib nor anti-c-fms antibodies affected the growth of eosinophil colonies in GM-CSF stimulated cultures until high (10.0μM) concentrations of imatinib were used. These results suggest that whereas GM-CSF directly stimulates eosinophil growth, it indirectly stimulates the growth of monocyte/macrophage colonies through autocrine production of M-CSF.

This hypothesis is supported by reports that GM-CSF stimulation of monocytes induces M-CSF protein secretion (Horiguchi *et al.* 1987; Gruber and Gerrard 1992). To examine the possibility that GM-CSF was acting indirectly in this present study, ELISA for M-CSF was performed on GM-CSF stimulated monocyte culture supernatants harvested over a 5 day period. The maximum level of M-CSF produced was estimated to be 70pg/mL, which is 300-500 fold lower than the concentration of added M-CSF used in experiments in this study. It is possible that a suboptimal concentration of M-CSF in combination with GM-CSF was sufficient to induce the growth and differentiation of monocytes, with GM-CSF acting synergistically to potentiate the effect of M-CSF.

In summary, this chapter demonstrates that imatinib inhibits the phosphorylation of c-fms at concentrations achieved therapeutically. These findings have major therapeutic implications and suggest that the clinical application of imatinib may be extended to include the treatment of diseases where abnormal c-fms activation is implicated, or diseases of monocyte/macrophage aetiology. This includes common cancers such as breast and epithelial ovarian cancer, and inflammatory conditions such as rheumatoid arthritis. The potent inhibitory effect of imatinib on c-fms phosphorylation also has important implications with regard to potential drug toxicity. Outside the haemopoietic system, c-fms signalling plays an important role in pregnancy, bone metabolism and inflammatory processes (Pollard 1997; Fixe and Praloran 1998). Although evidence to date demonstrates that imatinib is well tolerated by patients, potential effects of imatinib on these processes must also be considered as a consequence of long-term imatinib treatment. These issues are further discussed in Chapter 7.

Chapter 7:

GENERAL DISCUSSION

7.1 General Discussion

Chronic myeloid leukaemia (CML) is a haemopoietic malignancy that results from a reciprocal translocation between the long arms of chromosomes 9 and 22 to generate a hybrid gene bcr-abl (Rowley 1973; Groffen *et al.* 1984; Shtivelman *et al.* 1986). This bcr-abl gene is transcribed into a chimeric bcr-abl mRNA, and encodes a nonreceptor tyrosine kinase that displays constitutive and deregulated activity (Groffen *et al.* 1984; Konopka *et al.* 1985; Ben-Neriah *et al.* 1986). CML can involve myeloid, erythroid, megakaryocytic, monocytic and B cell lineages, and accounts for 15% of adult leukaemia cases (Faderl *et al.* 1999).

CML is associated with a progressive loss of the ability of the leukaemic clone to differentiate, and is characterised by 3 stages - chronic phase, accelerated phase and blast crisis. Diagnosis typically occurs during chronic phase, where elevated levels of white blood cells that retain their ability to differentiate are detected (Sawyers 1999). The disease invariably progresses to blast crisis within 3 to 5 years and, during this stage of CML, the cells fail to undergo complete maturation and resemble myeloblasts or lymphoblasts (Sawyers 1999).

Three mechanisms have been proposed to account for the malignant transformation in CML, namely reduced apoptosis (Bedi *et al.* 1994), altered adhesion (Gordon *et al.* 1987), and constitutively active mitogenic signalling (Puil *et al.* 1994; also reviewed in Deininger *et al.* 2000). Although these abnormalities are presumably mediated by bcr-abl, the resultant effect of bcr-abl on the growth characteristics of CML progenitor cells remains controversial. For example, the proliferation of CML progenitors has been demonstrated to be similar to that of normal progenitor cells (Bedi *et al.* 1994; Thiele *et al.* 1997), while other studies suggest that bcr-abl actively stimulates proliferation (Lepine and Messner 1983; Cortez *et al.* 1997; Jonuleit *et al.* 1998).

In Chapter 3, the proliferation and differentiation of $CD34^+$ progenitor cells from normal donors was compared to that of $CD34^+$ cells from 3 chronic phase CML patients using CFSE analysis. Decreased entry of cells into the cell cycle was observed on day 4 of culture in 2 CML patients, and the proliferation index was reduced in all patients. Analysis

of the distribution of cells across each division cycle on day 3 of culture suggested that cells from some patients were also arrested in their proliferation once they entered the cell cycle. By day 4 of culture, however, the distribution of cells across each division was similar in 2 of the 3 CML patients, suggesting that CML cells may have an increased stimulation threshold, but once this threshold is reached the cells retain the ability to undergo normal rates of proliferation. These results support the hypothesis that bcr-abl does not promote the entry of these cells into the cell cycle, and that the cells retain, at least in part, normal responses to proliferative and differentiative signals (Holyoake, 1999 #77, reviewed in Kabarowski and Witte 2000). Alternatively, variability in the response of $CD34^+$ cells from CML patients compared to cells from normal donors may be attributable to differences in the maturity of cells composing starting populations. This hypothesis is supported by the identification of differences in the phenotype of starting cells from CML patients at the initiation of the culture.

Identification of abnormalities in the differentiation of CML cells is hampered by our limited understanding of the factors that control the behaviour of HSC under normal conditions. In the second part of Chapter 3, the differentiation of $CD34^+$ cells was correlated with cell division to ascertain whether these properties were directly linked, and whether this relationship was perturbed in $CD34^+$ cells from CML patients. From these studies it was apparent that unlike the differentiation of B cells, where DNA rearrangement and deletion instigates a defined program of differentiation, the differentiation of normal $CD34^+$ was not directly related to cell division events. Because of this, defining a program of differentiation that occurs with cell division in $CD34^+$ cells is extremely difficult.

However, the expression of several markers including HLA-DR, CD38, CD13, CD33 and CD14 was found to be regulated by cell division alone in normal donors. In CML patients, marked variation in the expression of these proteins also occurred with the length of time the cells spent in culture. Differences in the percentage of cells expressing various phenotypic markers between normal donors and CML patients were apparent in early division cycles, however by division number 5 or 6, the percentage of cells expressing CD33 and CD36 was similar in normal and CML cultures. This was similarly observed for the level of CD38 expression and suggests that although CML $CD34^+$ cells may display

altered differentiation programs, chronic phase $CD34^+$ cells retain the ability to differentiate into mature myeloid cells. This finding further supports the hypothesis that CML $CD34^+$ cells largely retain normal responses to differentiative signals (Holyoake, 1999 #77, reviewed in Kabarowski and Witte 2000). It would be of benefit to extend these studies to include analysis of transcription factor expression concurrent with cell division history using CFSE analysis. Since modulation of transcription factor expression precedes protein expression, this technique would provide a more powerful tool by which aberrations to the process of differentiation following leukaemic transformation could be examined.

CML is almost uniformly fatal, except in instances where bone marrow transplants are possible. For those patients who are not suitable to receive bone marrow transplants, targeted therapy with the tyrosine kinase inhibitor, imatinib, is offering promising results. Imatinib is a novel therapy, as it is a rationally designed drug that competitively inhibits the ATP binding domain of bcr-abl. By inhibiting the phosphorylation of tyrosine residues on bcr-abl, the kinase activity is inhibited and the malignant cells are induced to undergo apoptosis or growth arrest (Druker *et al.* 1996; Deininger *et al.* 1997; Gambacorti-Passerini *et al.* 1997).

Although imatinib is generally a well tolerated therapy, there have been reports of myelosuppression such as grade 4 neutropenia (47% of patients) and grade 4 thrombocytopenia (73% of patients) following administration of 600mg imatinib daily (Druker *et al.* 2001; Sawyers *et al.* 2002; Verweij *et al.* 2003). This myelosuppression has been attributed to a pharmacological effect of imatinib on malignant cells, and a delay before normal HSC recover from suppression by the leukaemic clone (Sawyers *et al.* 2002). Since a mildly suppressive effect of imatinib on normal haemopoiesis has been observed *in vitro* (Deininger *et al.* 1997), the effect of imatinib on normal colony formation was further characterised in this present study to ascertain whether a suppression of normal haemopoiesis may contribute to this myelosuppression. These experiments were additionally performed to examine whether a particular cell type was inhibited by therapeutically relevant concentrations of imatinib and to potentially identify further targets of this drug.

Preliminary experiments described in Chapter 4 demonstrated an inhibition of colony growth in cells from the bone marrow of normal donors when treated with therapeutic concentrations of imatinib. Staining of cultures with non-specific esterases and luxol fast blue dye suggested that monocyte/macrophage colony growth was preferentially inhibited by imatinib, as neutrophil and eosinophil colony growth required 6-10 fold higher concentrations of imatinib for inhibition to be observed. Although a specific effect of imatinib on monocyte/macrophage development from $CD34^+$ progenitor cells was confirmed using an M-CSF and/or GM-CSF growth stimulus, colony assays could not identify the point of monocyte/macrophage development that was affected by imatinib. This led to experiments that specifically examined the effect of imatinib on monocyte cultures, and it was observed that therapeutic concentrations of imatinib inhibited the differentiation of monocytes into macrophages.

Macrophages play a vital role in the elicitation of immune responses. Their functions are manifold, and include activation of inflammatory processes following exposure to lipopolysaccharide on the surface of pathogens, antigen uptake by means of phagocytosis, and activation of adaptive immune responses following presentation of processed antigen to T cells. The observation that the maturation of monocytes into macrophages was inhibited by therapeutic concentrations of imatinib in Chapter 4 suggested that the ability of the cells to function immunologically may be also be compromised. Suppression of function was confirmed in Chapter 5, with induction of inflammatory responses to LPS, phagocytosis of antigen, and activation of T cell responses by cultured monocytes inhibited at therapeutic concentration of imatinib.

When taking these results into consideration with those from Chapter 4, it was apparent that a signalling pathway specifically involved in monocyte/macrophage development was being inhibited by imatinib. This hypothesis was supported by an inability to attribute the inhibitory effect of imatinib on monocyte/macrophage development and function to an inhibition of already identified imatinib targets such as c-kit and PDGF receptors. In particular, the inhibition was observed in the absence of added or endogenous SCF, suggesting that blockade of c-kit by imatinib was not responsible for the suppressive effect.

While a potential PDGF receptor-mediated mechanism was not directly ruled out, and PDGF can indirectly stimulate murine colony-forming unit-granulocyte-monocyte (CFU-GM) proliferation from bone marrow cells, PDGF has no effect on CFU-GM proliferation by $CD34^+$ cells (Yang *et al.* 2001). This may be related to the fact that PDGF receptors are not observed on freshly isolated $CD34^+$ cells (Su *et al.* 2002).

The M-CSF receptor, c-fms, plays a vital role in the growth and differentiation of the monocyte-macrophage lineage (Sherr 1990). As c-fms belongs to the same family of receptor tyrosine kinases as c-abl, c-kit and the PDGF receptors, it was surprising that imatinib was reported to not affect the tyrosine phosphorylation of c-fms up to a tested concentration of 10.0µM imatinib (Buchdunger *et al.* 2000). The possibility that imatinib inhibited c-fms signal transduction at therapeutic concentrations was therefore re-examined in this study, since the analysis by Buchdunger *et al.* (2000) was carried out on whole cell lysates from a fibroblastic cell line, and it was uncertain whether specific stimulation by M-CSF was performed. In this present study, the effect of imatinib on the phosphorylation of c-fms was examined on c-fms immunoprecipitates following specific receptor stimulation with saturating doses of M-CSF. Such stimulation is essential in order to examine specific effects of imatinib on c-fms phosphorylation, as c-fms is constitutively in the non-phosphorylated form.

The results presented in Chapter 6 demonstrated inhibition of c-fms phosphorylation at therapeutic concentrations of imatinib, indicating that the *in vitro* profile of imatinib can be extended to include the M-CSF receptor, c-fms. An imatinib concentration of 1.4µM was estimated to inhibit c-fms phosphorylation by 50%, which is lower than the potency observed for inhibition of other members of the type III receptor tyrosine kinase family such as Abl (IC_{50}= 0.025µM imatinib), c-kit (IC_{50} = 0.1µM imatinib) and the PDGF receptors (IC_{50}= 0.25µM imatinib) (Druker *et al.* 1996; Buchdunger *et al.* 2000). A concentration of 1.4µM imatinib is still within the therapeutic dose range, however, particularly since current therapeutic approaches to CML involves administration of 600-800mg of imatinib daily (Kantarjian *et al.* 2003), equivalent to an *in vitro* concentration in excess of 3.7µg/mL imatinib (le Coutre *et al.* 2004).

Following the commencement of this study and the publication of results from Chapter 4 (Dewar *et al.* 2003), other reports detailing a suppression of normal haemopoiesis by imatinib have emerged. In these studies, imatinib inhibited the proliferation of $CD34^+$ cells in a dose-dependent fashion (Bartolovic *et al.* 2003), and the differentiation of $CD34^+$ cells into dendritic cells was inhibited by 1.0-5.0μM imatinib (Appel *et al.* 2003). The effect of imatinib on these cells was not attributable to an abrogation of c-kit signal transduction, and the suppression of dendritic cell development was linked to inhibition of signalling through a family of transcription factors involved in the regulation of a variety of genes known as RelB (Appel *et al.* 2003; Bartolovic *et al.* 2003). It was also proposed that the inhibitory effect of imatinib may be due to inhibition of as yet unknown targets of imatinib (Bartolovic *et al.* 2003).

The receptor c-fms is expressed on bone marrow precursors committed to the monocyte/macrophage lineage, and is also observed on human monocyte-derived dendritic cells generated with GM-CSF and IL-4 (Sherr 1990; Rieser *et al.* 1998). The development and terminal maturation of dendritic cells was not found to be dependent on M-CSF however (Rieser *et al.* 1998), suggesting that an inhibition of c-fms signalling cannot account for the inhibitory effect of imatinib on dendritic cells reported by Appel *et al.* (2003). Further experiments using an anti-c-fms antibody should be performed, however, to confirm a lack of involvement of c-fms signalling in dendritic cell proliferation and development.

The results presented in Chapters 4-6 have major therapeutic implications, and suggest that imatinib should now be assessed for activity in diseases where abnormal c-fms signalling is implicated. This includes common cancers such as breast and ovarian cancer, and inflammatory conditions such as rheumatoid arthritis. Abnormal expression of c-fms has been demonstrated on a range of human carcinomas including tumours of epithelial origin, such as carcinomas of the breast, ovary, endometrium, lung, kidney and pancreas (Kacinski *et al.* 1991; Kacinski 1997; Sapi and Kacinski 1999). Furthermore, abnormal expression of c-fms in breast tumours and advanced epithelial ovarian carcinomas has been demonstrated to correlate with tumour cell invasiveness and adverse clinical prognosis, and M-CSF

produced by breast tumours has been implicated in the promotion of bone metastasis in breast cancer (Toy *et al.* 2001; Sapi 2004).

Carcinomas of the breast express wild type c-fms at levels similar to that observed on monocytes, and its expression is regulated by a range of hormones that include glucocorticoids and progestins (Kacinski 1997). The tumour cells also produce extremely large amounts of M-CSF, such that high ascitic levels of M-CSF are indicative of poor prognosis in patients with advanced disease (Kacinski 1997). Activation of c-fms by M-CSF may occur when c-fms and M-CSF are co-expressed by the tumour cells, or following synthesis of M-CSF by monocytes or fibroblasts present in the tumour stroma (Sapi and Kacinski 1999). The activation of c-fms in tumour cells has been demonstrated to stimulate tumour invasion following production of M-CSF by tumour cells or stromal elements (Kacinski 1997; Sapi and Kacinski 1999). For example, c-fms expressing lung cancer-derived cell lines cultured in the presence of M-CSF demonstrated a 12-fold enhancement in cellular invasion compared to cells cultured in medium alone, and invasiveness increased with a parallel increase in c-fms expression (Filderman *et al.* 1992).

In addition to using imatinib as an anti-cancer agent, the results from this study suggest that imatinib may also have potential in the treatment of inflammatory diseases of monocyte/macrophage aetiology such as rheumatoid arthritis. The pathogenesis of rheumatoid arthritis is poorly understood, but is known to involve synovial tissue proliferation associated with angiogenesis, as well as lymphocyte infiltration to the synovial tissue (reviewed in Clavel *et al.* 2003). A known biochemical event that plays a key role in the pathogenesis of rheumatoid arthritis is phosphorylation of tyrosine residues, and the function of many of the cytokines that activate synovial cells and fibroblasts in the synovial tissue are mediated by tyrosine kinase receptors (Williams *et al.* 1992).

The use of imatinib for the treatment of rheumatoid arthritis has been supported by recent studies where 3 patients with refractory rheumatoid arthritis were administered 200-400mg of imatinib daily for a period of 12 weeks. A favourable response to imatinib treatment was observed in all patients, including a decrease in tender joint count and swollen joint count (Eklund and Joensuu 2003). It was postulated that the favourable response to

imatinib in patients treated for rheumatoid arthritis was due to inhibition of c-kit expressed on the surface of mast cells, as high levels of SCF have been recorded in the serum and synovial fluid of rheumatoid arthritis patients, and c-kit is constitutively expressed on the surface of mast cells (Ashman 1999; Carsons *et al.* 2000; Eklund and Joensuu 2003).

In a further study, a patient suffering from rheumatoid arthritis was treated with imatinib for CML. The patient was treated with 300mg of imatinib for 8 weeks, and demonstrated complete remission of both diseases following imatinib treatment (Miyachi *et al.* 2003). In this study, the therapeutic effect of imatinib on rheumatoid arthritis was attributed to inhibition of PDGF receptor tyrosine kinases expressed on the surface of synovial cells and fibroblasts (Miyachi *et al.* 2003).

In addition to imatinib mediating a therapeutic effect in rheumatoid arthritis patients by abrogating signal transduction through of c-kit and PDGF receptors, the results presented in Chapters 4-6 suggest an additional mechanism may be through inhibition of c-fms. In a murine model of rheumatoid arthritis, daily injections of M-CSF exacerbated disease symptoms, and cells that were dependent on M-CSF were essential for the development of the chronic inflammatory disease suggesting that monocytes/macrophages play a vital role in the pathogenesis of rheumatoid arthritis (Bischof *et al.* 2000; Campbell *et al.* 2000). Furthermore, M-CSF has been detected in synovial fluid samples from patients with rheumatoid arthritis (Firestein *et al.* 1988).

While the results from this study potentially increase the clinical application of imatinib as a therapy for cancer and rheumatoid arthritis patients, inhibition of c-fms phosphorylation by therapeutic concentrations of imatinib also has important implications with regard to potential side effects. These findings suggest that the myelosuppression observed in patients currently receiving imatinib treatment may, in part, be attributable to an inhibition of monocyte/macrophage growth and differentiation as a consequence of an inhibition of c-fms signal transduction. Outside the haemopoietic system c-fms signalling also plays an important role in reproduction by regulating gonadal steroidogenesis and ovulation, and affecting pre-implantation embryo development and mammary gland development during pregnancy (Pollard 1997; Sapi and Kacinski 1999). In addition, c-fms plays an important

role in bone metabolism and inflammatory processes (Fixe and Praloran 1998). The effects of imatinib on these processes must also therefore be considered as a potential consequence of long-term imatinib treatment.

Chapter 8:

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THESIS AMENDMENTS

Corrections Relevant to Entire Thesis

- Error bars represent the SEM, and were derived from triplicate data points unless otherwise stated.
- Human genes should be written in capitals and italics. Proteins encoded by these genes should be written with the first letter in capital case, and should not be preceded by the "c-" prefix.

Chapter 1: Introduction

- Page 11, paragraph 1: the final sentence should be deleted as it does not follow.
- Page 14, paragraph 3: RAS is part of the MAPK pathway. The MAPK pathway is therefore not a further example of signal transduction pathways activated following ligand binding to the GM-CSF, IL-3 and IL-5 receptors.
- Page 26: The word "invariably" should be changed to read "usually", as there are many cases where CML patents remain in chronic phase for longer than 5 years.
- Page 28, first paragraph, final sentence: *Heistercamp et al (1990)* reference is incorrect. The reference should be *Daley et al (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome; Science. Feb 16; 247(4944): 824-30.*
- Page 31, 2nd paragraph: Reference to Deininger *et al* (1997) in statements that imatinib inhibits the growth of CML cells with minimal effects on normal haemopoiesis were omitted.
- Figure 1.1: LT-HSC is a "long-term" HSC. ST-HSC is a "short-term" HSC.

Chapter 2: Materials and Methods

- Page 46, 2.3.1: The final sentence in paragraph one should read: "Media *were* prewarmed...".
- Page 47, 2.3.1.1 & page 71, 2.5.5: The dilutions of IL-3 and GM-CSF that provided maximal cell growth were determined by titration using a factor dependent cell line.
- Page 71. 2.5.5: Use of the term selected means that the cells were selected for the ability to grow in M-CSF.

Chapter 3: Results Chapter 1

- Viability was determined following calculation of the percentage of cells with decreased FS/SS values.
- Surface markers were chosen which were expressed on specific myeloid lineages.
- Table 3.2: CML values were deemed significantly different from normal controls when p values calculated using ANOVA were less than 0.05.
- The data in Figures 3.13-16 are derived from the same experiment as Figures 3.5-3.8.
- Page 99, paragraph 1: "proliferative potential" should read "proliferation".

Chapter 4: Results Chapter 2

- Figure 4.2: The data was normalised relative to the 5HGF control, not the 4HGF control.
- Figure 4.9A (and elsewhere): Contrary to the statement in the legend, the marker tool was set on the 95th percentile of the fluorescence for the negative control.
- Figure 4.10: The morphology of the cells is not that of promyelocytes. The cells display an atypical morphology (excentric nucleus and a large, clear, vacuolated cytoplasm), however tristaining suggests the cells are immature myeloid cells.

Chapter 5: Results Chapter 3

Page 129, 5.2.3.2: The opsonin used was derived from purified rabbit polyclonal IgG antibodies that are specific for the zymosan particles. Phagocytosis of opsonsised zymosan particles was mediated by Fc and/or complement receptors.

Chapter 6: Results Chapter 4

Figures 6.6 and 6.7: "...infected with human c-fms..." should read "infected with a retrovirus encoding human FMS...".

Chapter 7: General Discussion

Page 152, paragraph 2: Abl is an intracellular tyrosine kinase, not a receptor tyrosine kinase.