GM-CSF protection of CML CD34$^+$ cells from the inhibitory effect of imatinib

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DECLARATION

This thesis contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institute 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. All work was undertaken by myself, with the exception of the MTS assays described in topic 4.3.5 which was performed with the collaboration by J Griffiths and me.

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Pongtep Viboonjuntra.

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ABSTRACT

Imatinib mesylate has become the front line therapy for CML. It inhibits the ABL tyrosine kinase activity of the causative oncogenic fusion protein BCR/ABL. The efficacy of imatinib therapy has been impressive, with a majority of patients achieving complete cytogenetic responses. However, to date imatinib therapy has been unable to completely eradicate the leukaemic clone in the majority of cases. This suggests that leukaemic stem cells may not be totally dependent on the activity of BCR/ABL, or that there may be other factors supporting the survival of leukaemic cells. Cytokines of the IL-3 family are involved in the survival and growth of myeloid haemopoietic cells. A number of the signals generated are in common with the pathways used by BCR/ABL and previous published studies showed that the IL-3 family member GM-CSF was found in elevated levels in CML patient serum. Therefore, the central aim was to test whether GM-CSF could modulate the response of CML progenitors to imatinib. In addition, a further aim was to examine CML cell production of GM-CSF. When CML CD34+ cells were exposed to imatinib, spontaneous BCR/ABL driven cell division was strongly inhibited, and cells became apoptotic. However, the addition of GM-CSF reversed this effect, returning cell division and survival back to spontaneous levels. GM-CSF specificity was confirmed using the antagonist E21R. The cell division and survival of leukaemic CD34+ cells in cultures of total CML mononuclear cells were less sensitive to imatinib inhibition. However, inclusion of E21R in such cultures resulted in a similar pattern to CD34+ CML cells cultured alone. This suggested that non-CD34+ cells may be a source of GM-CSF. This was confirmed by sorting CD34+ and non-CD34+ CML cells and examining GM-CSF production, which was markedly more apparent in the non-CD34+ compartment. Finally, the effects of GM-CSF on the survival and proliferation pathways induced by BCR/ABL were examined, showing it may exert its effects through p-Erk and p-Akt mediated mechanisms. Our data suggest that GM-CSF can signal via pathways which are normally suppressed due to alternative activation of major substrates by BCR/ABL, as well as others which are not activated by BCR/ABL, leading to cell survival when kinase activity is blocked by imatinib.

ABBREVIATIONS

4X, 30X, 150X	4 times, 30 times, 150 times
2×10^5	2 times 10^5
7-AAD	7-aminoactinomycin-D
Akt	a serine/threonine kinase, also known as protein kinase B
a.a.	Amino acids
AI	Apoptotic Index
β_C	Common beta chain
µg	Microgram
µm	Micrometre
µM	Micromolar
4HGF	Four haemopoietic growth factors (IL-3, IL-6, FLT3-ligand, TPO)
6HGF	Six haemopoietic growth factors (IL-3, IL-6, G-CSF, SCF, FLT3-ligand, TPO)
ATP	Adenosine triphosphate
BAD	Bcl-XL/Bcl-2 associated death promoter
BAX	Bcl-2 associated X protein
BCR	Breakpoint cluster region
Bcl-2	B-cell lymphoma 2

Bcl-XL	A Bcl-X isoform that inhibits apoptosis
Bim	Bcl-2 interacting mediator of cell death
BM	Bone marrow
BSA	Bovine serum albumin
c-ABL	Abelson murine leukemia virus human homologue
Caspase	Cysteinyl aspartic acid protease
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CML	Chronic myeloid leukaemia
Crkl	Crk-oncogene like protein
CSF	Colony stimulating factor
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Erk	Extracellular signal related kinase
FACS	Fluoresceine activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Flurescein isothiocyanate
FLICA	Fluorochrome Inhibitor of Caspases

FOXO	a member of the forkhead family of transcriptional regulators (previously termed FKHR-L1)
g	Gram
GAP	Guanosine Triphosphate activating function
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
Grb2	Growth factor receptor-bound Protein 2
HBSS	Hank's balance salt solution
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
IMDM	Iscove's modification of Dulbecco's medium
JAK	Janus kinase
Jun	Jun kinase
kDa	Kilodalton
LC-8	Dynein light chain
M	Molar
m	Murine
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
mg	Milligram

mL	Millilitre
MNC	Mononuclear cell
ng	Nanogram
PB	Peripheral blood
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
Ph chromosome	Philadelphia chromosome
PI	Proliferation index
PI3-K	Phosphatidylinositol-3- kinase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase c
PPK	Phosphorylase kinase
R	Receptor
Rac	a Ras-like GTPase
RT	Room temperature
RPMI	Roswell Park Memorial Institute
SCF	Stem cell factor
SDM	Serum deprived medium
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Shc	Src homology 2- containing protein

STI571	Signal transduction inducer 571
TPK	Tyrosine protein kinase
TPO	Thrombopoietin
VEGE	Vascular endothelial growth factor
V/v	Volume per volume
W/v	Weight per volume

PRESENTATIONS AND PUBLICATIONS

Manuscripts:

P Viboonjuntra, J Griffiths, V Rozenkov, AF Lopez, TP Hughes and AB Lyons. GM-CSF modulates the effect of imatinib on proliferation and survival of CML $CD34^+$ cells. (submitted)

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AB Lyons, TP Hughes and P Viboonjuntra (2005), Production of GM-CSF by CML cells can modulate the anti-proliferative and pro-apoptotic effects of imatinib on CML $CD34^+$ cells. 47^{th} meeting of the American Society of Hematology, Atlanta, Georgia, USA

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Chapter 1.

Introduction

1.1. *Preface*

Chronic myeloid leukaemia (CML) is a clonal haematopoietic malignancy of stem cells. It is characterized by a biphasic clinical course. The initial chronic phase has an abnormal amplified clone that produces functionally normal, mature blood cells of all types except for those of the T-lineage (Fialkow, *et al* 1977). However, the hallmark of this disease in chronic phase is the markedly elevated number of granulocytes which results in development of disease symptoms. The spectrum of cells both in bone marrow and peripheral blood in the initial phase are similar, then as the disease progresses, immature cells become predominant. The progressive clinical course resembles acute leukaemia and is known as the blast crisis phase. More than a century after the identification of this disease, its annual incidence is quite stable, suggesting the genetic abnormality occurs by chance. However, this disease has higher incidence in the fifth decade of age. To date, because of better screening techniques, this disease is found in younger people than before, but the annual incidence remains the same.

Our understanding of the clinical, biological, biochemical and molecular features of CML has developed markedly since the disease was first described leading to the development of more specific chemotherapeutic approaches. Although the current treatment with small molecule inhibitor therapy is very effective, the abnormal clone remains in the bone marrow, even in patients after several years of treatment with imatinib. Complete eradication of leukaemic stem cells may require a more sophisticated approach to treatment.

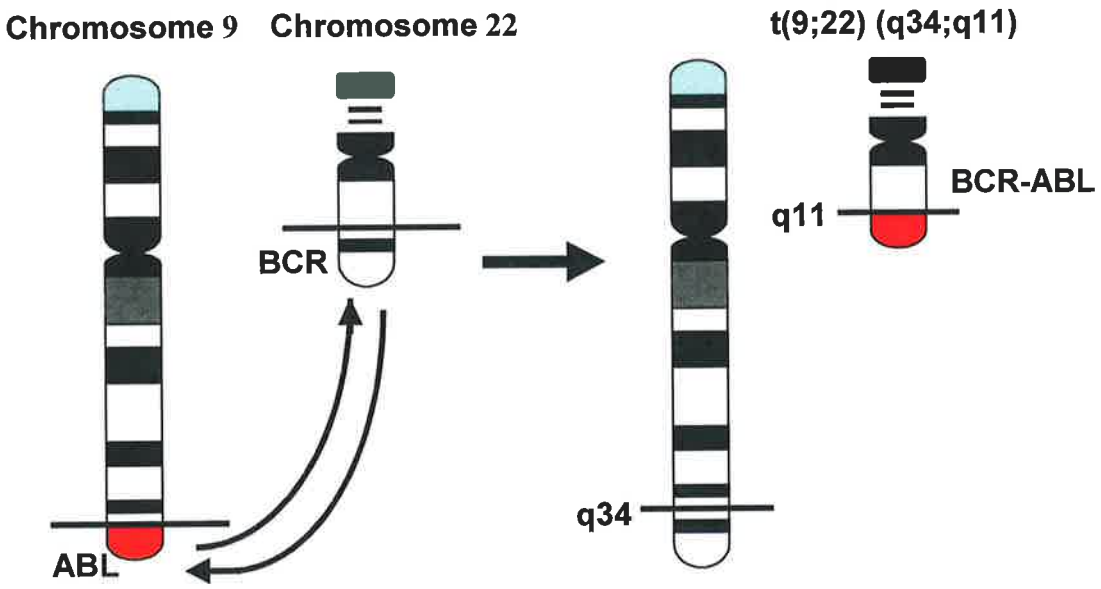
1.2. *History of chronic myeloid leukemia*

CML was first described in 1845 by 3 persons (Bennett 1845, Craigie 1845, Virchow 1845) who found a few cases presenting the same clinical signs including splenomegaly, anaemia and massive granulocytosis. Bennet called this massive white-blood cell accumulation in blood circulation as "Leucocythemia" because it was believed at the time that this condition had a correlation with infection. This disease was first termed "myelogenous leukaemia" (Neumann 1878) after no infectious clue was identified to cause the massive granulocytosis as previously suspected (Craigie 1845), and Neumann

Figure 1.1. The translocation between chromosome 9 and 22

Over 100 years elapsed from the first description of CML in 1845 until the identification of the frequent incidence of a shortened chromosome 22, or Philadelphia-1 chromosome, by Nowell and Hungerford in 1960.

In 1973, Rowley demonstrated with improved cytogenetic banding techniques that the Ph-1 chromosome results from a reciprocal translocation between chromosome 9 and 22.



Adapted from Faderl *et al* 1999

Figure 1.1

(Neumann 1878) noticed that this disease originated in bone marrow, but it took almost 80 years before the Ph 1-chromosome was discovered and led to a better understanding of the pathogenesis of the disease (Nowell and Hungerford 1960). The observation of the frequent incidence of a shortened chromosome 22 or Ph-chromosome(Nowell and Hungerford 1960) led to the close examination of the cause of this cytogenetic feature in 1973 by Rowley using an improved chromosome banding technique (Rowley 1973) (Figure 1.1). It was then determined that a translocation between chromosome 9 and 22 formed the minute Ph 1- chromosome. This led to the identification of the BCR/ABL fusion oncogene in the 1980s which was found to encode the $p210^{BCR/ABL}$ protein (Figures 1.2 and 1.3). This protein was eventually found to transform primary myeloid cells and induce CML-like manifestation in mice (Daley, *et al* 1990, Gale, *et al* 1998, Kelliher, *et al* 1990, Pear, *et al* 1998). This abnormal chromosome is found in 90-95% of patients who present clinical features of CML (Clarkson, *et al* 2003, Daley and Ben-Neriah 1991, Kurzrock, *et al* 1988).

1.3. *Clinical overview and therapy of chronic myeloid leukaemia*

1.3.1. *Epidemiology*

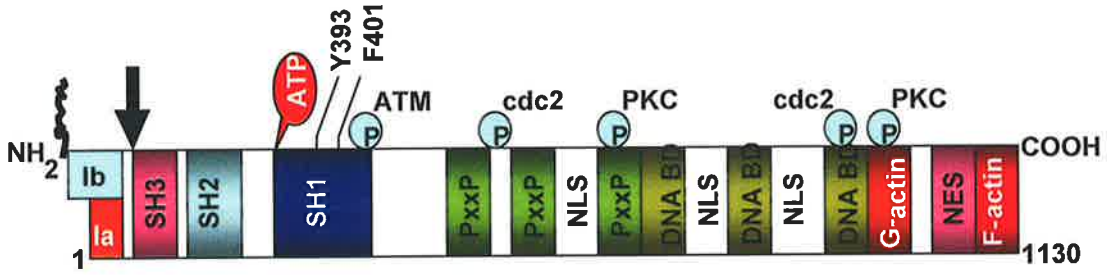
According to the latest US statistics, CML patients comprise 14% of all leukaemia cases. The annual incidence of CML is 1-1.6 cases per 100,000 people (Faderl, *et al* 1999, Sawyers 1999, Silver, *et al* 1999), and this number is quite constant worldwide. Male to female ratio is 1.4-2.2:1 (Cortes, *et al* 1996), but the clinical course is similar in both sexes (Cortes, *et al* 1996). The median age at diagnosis of CML patients is 55-60 years with less than 10% of cases under age 20 years, but the disease occurs in all age groups(Cortes, *et al* 1996). The constant number of the annual incidence suggests that the leukaemic translocation occurs randomly. The only known risk factor for this disease is a high dose of ionizing radiation. Reports on survivors of atomic disasters at Nagasaki and Hiroshima (Ichimaru, *et al* 1978) and on some patients who received therapeutic radiation for ankylosing spondylitis (Court Brown and Doll 1959, Court Brown and Doll 1960) and uterine cervical cancer (Boice, *et al* 1985) were also shown to have increased risk of CML. There is little evidence for inheritance of CML because no higher incidence is found among the offspring of the parents with CML than the general population and also no

Figure 1.2. The normal two dimensional block structure of the protein c-ABL (p-145) and protein BCR (p-160).

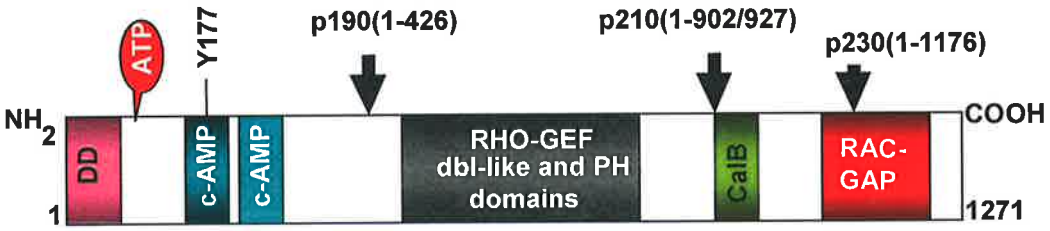
Panel a, normal p-145 ABL showed the complete range of this protein which comprises of the myristoylation site (1b) at the NH_2 terminus. This domain plays a role in the attachment of this protein to the plasma membrane, and will be lost due to the BCR/ABL translocation or when the normal ABL gene encodes the 1b isoform. The site of frequent fusion event during translocation is shown by the black arrow. SH-1 is the tyrosine kinase domain which is inhibited by the SH-3 domain in normal c-ABL. The PxxP domain is the proline-rich motif which is capable of binding to the SH-3 domain. The three NLS's - nuclear localisation signals and an NES - nuclear exporting signals play a role in the ABL shuttling. The COOH-end comprises the G and F actin binding domains.

Panel b, normal BCR shows the dimerisation domain which contains the coiled-coil motif domain at the far end of the NH_2-terminal. The coiled-coil motif is important for the dimerisation of BCR with other proteins such as BCR and BCR/ABL in BCR/ABL positive cells and promotes interactions including the phosphorylation of recruited proteins. The black arrow shows the site of fusion with ABL during translocation.

a. p145 ABL



b. p160 BCR



Adapted from Deininger *et al.*, 2000

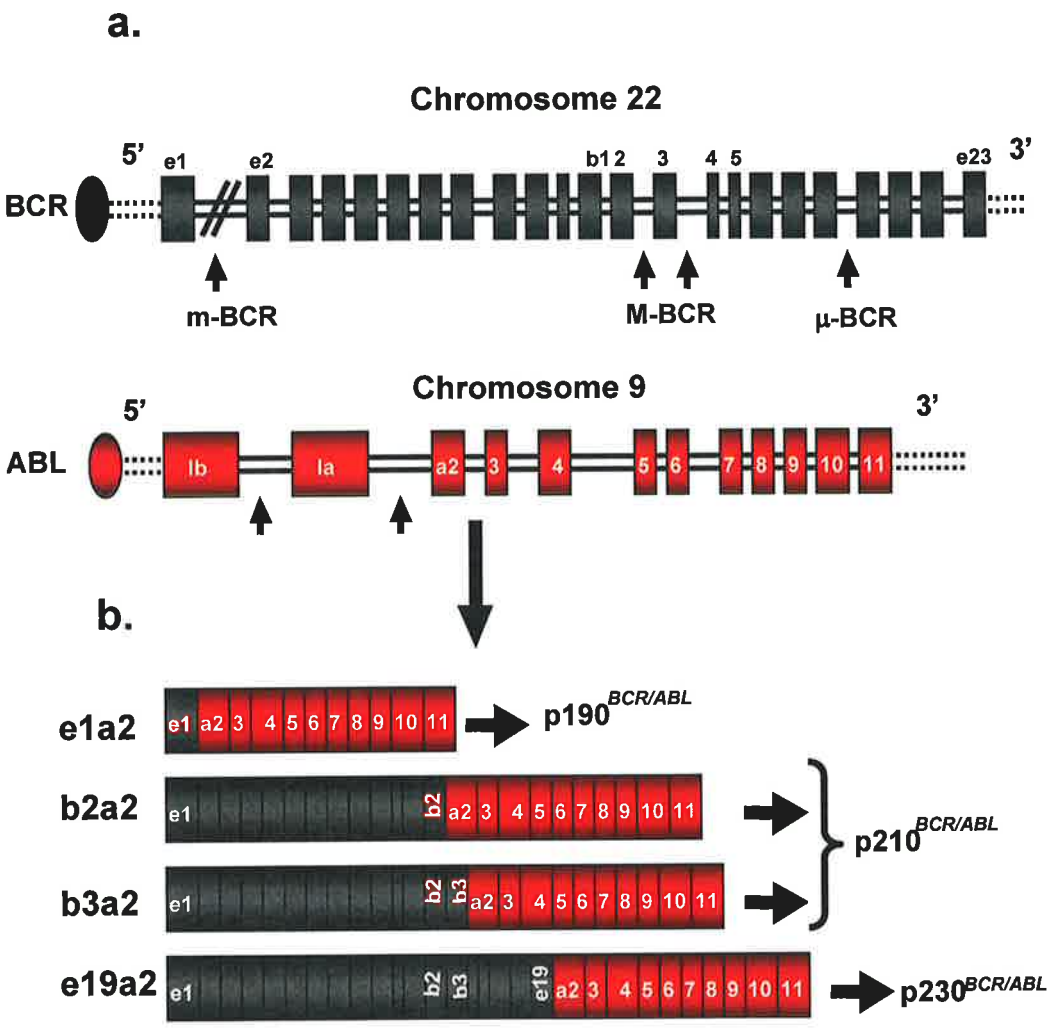
Figure 1.2

Figure 1.3. Diagram showing the fusion transcripts found in CML and Ph-positive ALL.

Panel a, the BCR gene comprised of 13 exons upstream of the M-BCR region (grey boxes at e1 side), which is comprised of exons b1 to b5, and 7 exons downstream of M-BCR (grey boxes at e23 side). Breakpoints in CML usually occur between exons b1 and b2 or between exons b2 and b3 of the M- BCR.

The ABL gene contains two alternative 5' exons (named 1b and 1a) followed by 10 common exons numbered a2 to a11 (shown by red boxes). Breakpoints in CML and Ph-positive ALL usually occur in the introns between exons 1b and 1a or between exons 1a and a2 (as shown by vertical arrow).

Panel b, the three possible BCR/ABL mRNA transcripts are shown below. The first mRNA (e1a2) is found in the majority of patients with Ph-positive ALL. The second 2 m-RNAs (b2a2 and b3a2) are characteristic of CML. The bottom mRNA (e19a2) is rare characteristic type found in chronic neutrophilic leukaemia (CNL).



Adapted from Deininger *et al.* 2000

Figure 1.3

correlation is found in monozygotic twins. However, CML has been found to correlate with HLA antigens CW3 and CW4 (Bortin, *et al* 1987) with the relative risk = 2.07 and 2.14 respectively.

1.3.2. *History of CML treatment*

The classic natural history of CML is a triphasic course. Over 80% of patients are still in the chronic phase at diagnosis and the median survival of patients in this phase is around 35 to 65 months (Hehlmann, *et al* 1993). Most patients will progress to a blast crisis with the median survival of 3 to 12 months if the disease cannot be suppressed or eliminated before entering the blastic phase (Faderl, *et al* 1999). Two thirds of patients will enter an intermediate phase of which the clinical spectrum is between the chronic and blastic phases, also known as accelerated phase, with the median survival of 1 to 2 years (Faderl, *et al* 1999). Treatment recommendation was always provided in the chronic phase because of its least aggressive stage.

1.3.2.1. *Busulfan and Hydroxyurea*

Previously, treatment recommendations for CML were not defined because of a lack of statistically comparative evidence (Silver, *et al* 1999). Historically, busulfan was first reported to be used in 1953, and became the treatment of choice for CML in 1958 (Silver, *et al* 1999). However, the survival advantage of busulfan treatment compared with splenic irradiation, ^{32}P, and no treatment was not statistically different (Hehlmann, *et al* 1993). Busulfan causes serious side effects including infertility, adrenal insufficiency and risk of marrow aplasia and lung fibrosis (Silver, *et al* 1999). Hydroxyurea became popular in the late 1960s and remains a mainstay of palliation in CML up to now, because of fewer side effects than busulfan (Silver, *et al* 1999). Hydroxyurea is a ribonucleotide reductase inhibitor, and the treated patients are expected to display macrocytosis and hypersegmented polymorphonuclear cells (Silver, *et al* 1999). Hydroxyurea occasionally causes skin rash and gastrointestinal symptoms (Silver, *et al* 1999). These two reagents are now used exclusively for cytoreductive therapy because neither busulfan nor hydroxyurea induces cytogenetic responses (Hehlmann, *et al* 1999, Ohnishi, *et al* 1998) and both can

change neither the median survival of the chronic-phase patients, nor the onset of blast crisis.

1.3.2.2. *Interferon-α*

The most successful CML therapy apart from allogeneic transplantation prior to the development of targeted small molecule tyrosine kinase inhibitors is interferon-α. Interferon-α, a glycoprotein, is produced by leukocytes in response to stress. It became the treatment of choice in patients with Ph-positive CML who were not candidates for allogeneic stem cell transplantation. It's possible mechanisms include affecting multiple gene transcription and protein phosphorylation events (Galvani and Cawley 1989, Gordon, *et al* 1987, Sawyers 1999), activation of dendritic cells (Choudhury, *et al* 1997), affecting Fas-R-mediated induction of apoptosis (Choudhury, *et al* 1997, Selleri, *et al* 1998), downregulating BCR/ABL mRNA transcription and p210 production, and suppressing cell growth and inducing apoptosis via cooperative interaction of ICSBP (interferon consensus sequence binding protein, a member of IRF family transcription factor, and PU1 (an ets family transcription factor) on the regulation of bcl-2 gene expression (Burchert, *et al* 2004, Chen, *et al* 2002).

Interferon-α was found to result in haematological responses in 70% to 80% of CML patients and complete cytogenetic responses in 6% to 26% of patients (Talpaz, *et al* 1991) as a single agent. Randomised clinical trials showed that interferon-α could prolong median survival up to 20 months (about 65-90 months) and an absolute advantage of 15% at 5 years reported by the Benelux CML Study Group (1998). Patients who achieve a complete cytogenetic response have a survival probability of 78% at 10 years (1994, Hehlmann, *et al* 1994, Kantarjian, *et al* 1999, Kantarjian, *et al* 1995). In the majority of patients who had complete cytogenetic responses, a small number of leukaemic cells were still detected by using PCR technology or FISH analysis (Eberle, *et al* 1995, Hochhaus, *et al* 1995, Lee, *et al* 1992, Opalka, *et al* 1991). However, 14 to 26 percent of patients had to discontinue interferon-α because of its side effects, including fatigue, myalgia, arthralgia, headache, weight loss, depression, diarrhoea, neurological symptoms and memory changes, hair thinning, autoimmune disease and cardiomyopathy (1998). Interferon-α is

more effective in inducing both haematological responses and cytogenetic responses than either busulfan or hydroxyurea. Some studies suggested that the combination of interferon-α and cytarabine might be more effective (1998, Guilhot, *et al* 1997). This combination can improve the response rate but the patients encounter increased side effects, such as greater haematological, gastrointestinal and mucosal toxicity, and also leads to the termination of treatment due to intolerance to these side effects (1998, Guilhot, *et al* 1997).

1.3.2.3. *Chemotherapy and Bone marrow transplantation*

Chemotherapy alone cannot induce sufficient cytogenetic remission in CML. The approved therapy which is, to date, most effective is allogeneic stem cell transplantation. Three year survival probabilities of 78% have been reported (Radich, *et al* 2003). However, the information regarding long-term follow-up with bone-marrow transplantation is very little. Approximately 50-60% of patients in chronic phase who were treated with allogeneic bone marrow transplants gained long-term survival, usually > 5-7 years (Gale, *et al* 1998, van Rhee, *et al* 1997). However, this therapy is only available to less than 40% of patients because of the age of the patients and the limitation of number of donors (Verfaillie, *et al* 1999). Thus, experimental approaches are needed to improve the survival in the majority of patients where no effective therapy currently exists.

1.3.2.4. *The tyrosine kinase inhibitor imatinib*

The tyrosine kinase activity of BCR/ABL protein is crucial for cell transformation of CML cells. Therefore the rational approach for treatment of this disease has focused on the tyrosine kinase region of this molecule. The 2-phenylaminopyrimidines, a series of compounds based on the known structure of the ATP binding site on the ABL-tyrosine kinase domain (SH1) was reported to selectively inhibit the activity of ABL (Buchdunger, *et al* 1996, Buchdunger, *et al* 1995). Imatinib (formerly CGP57148 or STI571, commercial named Gleevec or Glivec, Novartis) was derived from this series of molecules and successful clinical trial has changed the strategy of treatment of CML. Although imatinib can inhibit other tyrosine kinases such as PDGF-R and c-Kit (Buchdunger, *et al* 1996, Buchdunger, *et al* 1995), this reagent is specific enough to eliminate the CML-leukaemic cells with only mild toxicity to normal progenitor cells. Toxic effects on cell

lines not expressing BCR/ABL are not seen until the concentration of imatinib reaches 10μM (Deininger, *et al* 1997). The myelosuppression is the expected result from the suppression of the growth of huge number of leukaemic cells which will be restored by the normal cells after 2-weeks of treatment (Marin, *et al* 2003). This tyrosine kinase inhibitor molecule, STI 571, has been conducted in Phase I/II clinical studies in June 1998 targeting advanced phase CML, Ph^+ ALL, acute myeloid leukaemia and chronic phase CML refractory to, or intolerant of interferon (Druker and Lydon 2000, Druker, *et al* 2001). A dose response relationship to imatinib was shown in an early trial with the cytogenetic response rate of 54% at doses of 300-1000 mg daily and less than 10% at doses less than 300 mg daily. No dose-limiting toxicity was encountered and the side-effects have been tolerated after dose escalation to 1000 mg daily (Druker and Lydon 2000, Druker, *et al* 2001). These preliminary studies suggested imatinib was a superior treatment of CML. However, in one series of 268 patients in chronic phase who achieved a complete cytogenetic remission with imatinib therapy at 300mg to 800 mg/day, only 76 patients reached undetectable levels of BCR/ABL by quantitative and nested PCR (Cortes 2003). This is quite interesting that the leukaemic clones are still preserved despite treatment with imatinib. It may be other mechanisms or endogenous factors apart from the mechanisms through BCR/ABL that preserve these leukaemic clones, such as the influx/efflux mechanism which leads to insufficient concentration of imatinib or the stimulation of the substrates downstream to BCR/ABL by the endogenous/exogenous cytokines to help the CML progenitors to survive imatinib.

1.4. ***Molecular pathophysiology of CML***

The BCR/ABL or $p210^{BCR/ABL}$ protein has uncontrolled tyrosine kinase activity and abnormal cellular localization (Clarkson, *et al* 2003), which are the two important elements governing the transforming potency of the BCR/ABL protein (Clarkson, *et al* 2003, Konopka and Witte 1985, Van Etten, *et al* 1989). The normal $p145^{ABL}$ protein is thought to shuttle between the cytoplasm and the nucleus, and to bind specifically to DNA (Clarkson, *et al* 2003). In contrast to c-ABL, the BCR/ABL or other ABL transforming proteins are only in the cytoplasm and lack the ability to bind DNA (Clarkson, *et al* 2003). The function of ABL, normal BCR, and BCR/ABL will be described in the next sections.

1.4.1. *Normal p145^{ABL} protein*

The normal c-ABL protein or p145^{ABL} is classified in the family of non-receptor tyrosine kinases, and contains several defined domains. This protein has 2 isoforms namely 1a and 1b because of random alternative splicing of two distinct first exons on the c-ABL gene (Fainstein, *et al* 1989). The 1a isoform has 1130 a.a. whilst 1b isoform contains an additional 19 a.a. owing to the longer length of exon 1b (45 a.a.) than the alternate exon 1a (26 a.a.) (Fainstein, *et al* 1989). Interestingly, 1b exon encodes a myristoylation site at the NH_2-terminal glycine residue of 1b-p145^{ABL} (Ben-Neriah, *et al* 1986, Fainstein, *et al* 1989). This site is important for the localization of 1b isoform to the inner plasma membrane after myristoylation (Figure 1.2).

Conventional protein tyrosine kinases (PTKs) possess catalytic domains called SH1, the tyrosine kinase domain, typically located near the carboxy-terminal of the molecule (Hanks, *et al* 1988). Apart from SH1, other characteristic PTKs motifs enable the kinases to interact with the diverse signaling intermediates include the SH2, SH3 domains, the pleckstrin homology domain (PH), and a negative regulatory tyrosine in the carboxy terminus and myristoylation (such as 1b isoform of c-ABL) or palmitoylation (other Src familiy members) lipid modification sites at the N-terminus (Cooper and Howell 1993, Pawson 1995). The PH domain and lipid modification sites apart from myristoylation domains are also important for the attachment of the kinases to the plasma membranes.

The Abelson virus oncogene v-ABL is derived from recombination of c-ABL gene with the viral Gag gene in which the viral Gag sequence also provided a myristoylation signal and causes v-ABL to localize predominantly at the plasma membrane (Jackson and Baltimore 1989). In addition, the SH3 domain in the NH_2-terminal half of c-ABL is replaced by viral sequence. This confirms that the myristoylation site can induce 1b-isoform c-ABL to localize adjacent to the plasma membrane (Rosenberg 1982). However, the loss of the SH3 domain, which is normally the negative regulatory domain for tyrosine kinase activity of c-ABL (Prywes, *et al* 1983), also enhances the tyrosine kinase activity of v-ABL. This enhancement recruits the structural protein F-actin and causes focal adhesion of v-ABL to the plasma membrane. In addition, the NH_2 terminal half of p145^{ABL} contains the Src-homology (SH) regions 1, 2 and 3 that mediate tyrosine kinase activity on SH1,

phosphotyrosine protein binding (SH2), and kinase inhibitory (SH3) functions (Ben-Neriah, *et al* 1986). The carboxy-terminal half (COOH) of this protein has a lysine-rich motif required for nuclear localization (Van Etten, *et al* 1989), DNA binding domain (Kipreos and Wang 1992) and F/G actin binding domains (McWhirter and Wang 1993, Van Etten, *et al* 1994). The lysine rich motif contains three nuclear localization domains (NLS) (Wen, *et al* 1996) and a nuclear export domain (NES) (Taagepera, *et al* 1998). Both NLS and NES are important for the nuclear-cytoplasmic shuttling of the ABL protein. In addition, in the normal situation without any stimulation, it has been found that only 30-40% of c-ABL is localised in nucleus as measured using digital quantification by deconvolution microscopy (Vigneri and Wang 2001). After treatment with leptomycin B a inhibitor of nuclear export, the nuclear retention of ABL was increased to 60-70% (Vigneri and Wang 2001). This suggests that c-ABL shuttles between nucleus and cytoplasm and it has apparently no obvious function in non-stressed cells. However, no studies have determined whether or not the 1b isoform is stably localised in cytoplasm. The myristoylation and F-actin binding site may only maintain cytoplasmic localization of ABL, and have no role in ABL shuttling and activation of the tyrosine kinase activity of c-ABL. Also, how cytoplasmic p145 is activated is not clearly understood. The function of cytoplasmic p145 is observed in only the mutated ABL or ABL- related proteins such as v-ABL or BCR/ABL *in vitro*. Similarly, the v-ABL and 1b isoform p145 are localized in cytoplasm because of their intact myristoylation sites (Rosenberg 1982). In contrast to the 1b isoform, v-ABL lacks the SH3 domain (Prywes, *et al* 1983), therefore it has enhanced auto-phosphorylation due to the up-regulate tyrosine kinase activity of the SH1 domain. This also causes cytoplasmic v-ABL to bind to F-actin and participate in signal transduction pathways that stimulate mitogenesis. Normal cytoplasmic p145 or 1b isoform may sometimes be activated and is believed to stimulate signaling pathways and is maintained in the cytoplasm through F-actin as well as via the myristoylation site. It is unclear which proteins regulate the SH3 domain of normal p145. Otherwise the activation of cytoplasmic ABL may occur through other domains rather than through the inhibition of SH3 domain. It is known that proline-rich motifs are involved in the binding to SH3 domains (Morton and Campbell 1994), and cause the maintenance of c-ABL in a conformation called the 'Fold-back' model which will suppress the interaction between c-ABL and other substrates (Goga, *et al* 1993). By using the 'Fold-back' mechanism,

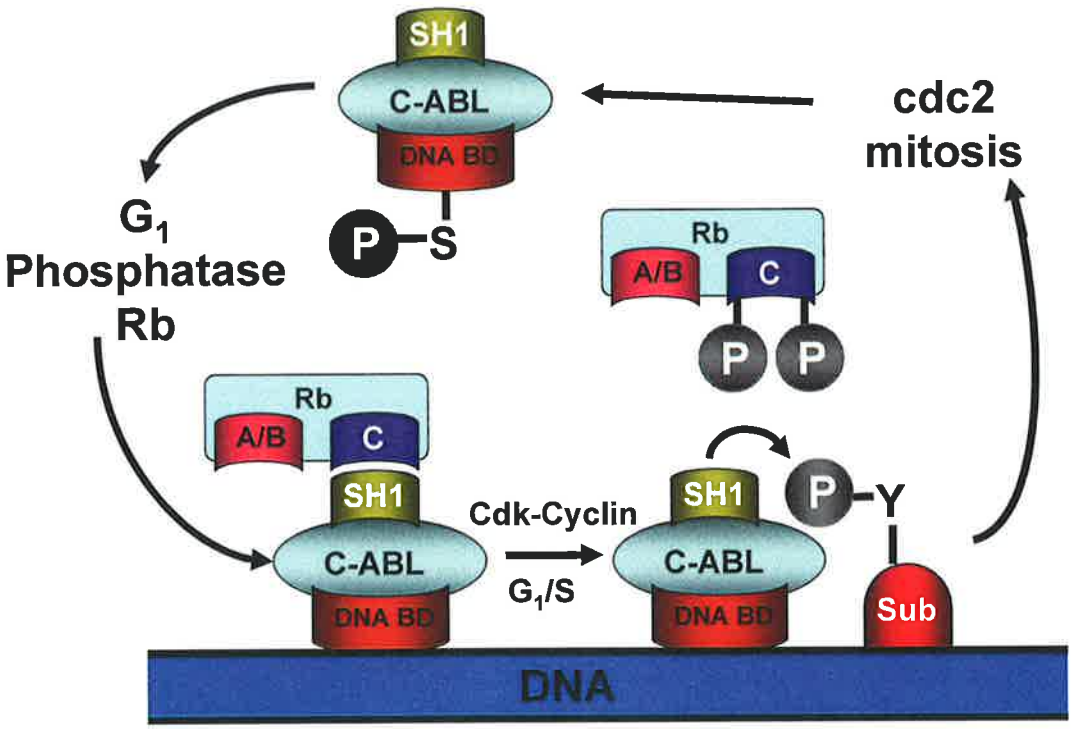
apparently in the normal situation no other proteins could unfold the binding of the SH3 domain to proline-rich motifs except the G1-phosphatase Rb protein which will allow the c-domain of Rb protein to bind the ABL ATP binding site (Goga, *et al* 1993, Verfaillie 1998) (Figure 1.4). The 'Fold-back' theory also suggests that the 1b isoform should have very little function on any signaling pathway, and v-ABL differs from c-ABL in the inhibition of the SH3 domain, which in turn will unfold the 'Fold-back' structure and cause uncontrolled tyrosine kinase domain SH1 activation.

This 'Fold-back' model is also observed in the negative regulation of the tyrosine kinases of the Src-family, in which the internal binding of the SH2 domains to their carboxyl terminal $pTyr^{527}$ residues usually occurs (Cantley, *et al* 1991). It is also unclear whether the nuclear and cytoplasmic pools of $p145^{ABL}$ belongs to a common pathway of cell regulation or serve different functions (Wang 1993). It has been reported that p145 ABL is predominantly localised in the nucleus rather than in the cytoplasm, and interestingly it is a positive inducer of apoptosis in response to DNA damage (Gong, *et al* 1999).

The nuclear c-ABL is regulated in a cell cycle dependent manner. The c-ABL is bound to DNA during interphase and is phosphorylated by the cdc2 kinase, which causes the dissociation of c-ABL from DNA during mitosis (Kipreos and Wang 1992, Wang 1993). In the steady state, c-ABL binds to Rb protein in a complex at the ATP binding site of c-ABL, and also binds to DNA via a DNA binding site of c-ABL (Verfaillie 1998). Once cells enter the cell cycle, Rb protein is phosphorylated and disrupted from c-ABL by cyclin dependent kinases at the G1/S interface (Figure 1.4). The active c-ABL which is now free from Rb protein may then phosphorylate the adjacent proteins that are involved in the regulation of gene transcription at the onset of S-phase (Wang 1994). For example, DNA - dependent RNA polymerase II (Pol II) can be phosphorylated *in vitro* in its carboxyl-terminal repeated domain by c-ABL and this may be one mechanism where c-ABL can influence gene transcription (Baskaran, *et al* 1993). Interestingly, overexpression of c-ABL can induce cell cycle arrest in susceptible cells suggesting its possible role in a negative regulator of cell growth (Sawyers, *et al* 1994). Mice homozygous for a null mutation in c-ABL appear lymphopenic, runted and die in the perinatal period (Schwartzberg, *et al* 1991). However, heterozygous mice appear normal. Therefore, c-ABL is essential for normal growth and development.

Figure 1.4. The function c-ABL involves cell cycle regulation.

The c-ABL is localised mostly in the nucleus in a cell cycle-dependent manner. Nuclear c-ABL is bound to DNA during interphase, and is inhibited in G1 by the binding of the c-domain of Rb to the ABL ATP binding site. Phosphorylation of Rb by the Cdk-cyclin (cyclin-dependent kinase) occurs at the G1/S interface inhibit binding of Rb to c-ABL and depression the ABL kinase. The active DNA-bound ABL will then activate the adjacent protien substrates which are involved in DNA transcription activity.



Adapted from Wang *et al.* 1994

Figure 1.4

1.4.2. *The normal BCR protein*

The BCR gene on chromosome 22 contains 23 exons over 135 kb of DNA. It encodes the 160-kDa BCR protein (Laneuville 1995). The function of normal BCR is still unclear. In the steady state, the BCR protein is localised in the cytoplasm in quiescent cells. The presence of this protein in a perichromosomal location during mitosis suggests a possible role for this protein in cell cycle regulation (Voncken, *et al* 1995).

The first 63 amino acid of BCR contains a coiled-coil motif which is important for dimerisation (McWhirter, *et al* 1993). Although the actual function of this protein in normal cells is unknown, the dimerisation motif is required for malignant transformation by the BCR-ABL protein (McWhirter, *et al* 1993). Exon 1 of BCR contains the serine-threonine kinase domain (Maru and Witte 1991, Pendergast, *et al* 1991) which binds to BCR associated protein-1 (BAP-1), a member of the 14-3-3 group of proteins(Aitken, *et al* 1995, Reuther, *et al* 1994). The 14-3-3 family proteins are highly conserved molecules that can sequester serine-threonine kinase activity of other proteins and play roles in signal transduction and cell cycle regulation (Aitken, *et al* 1995, Braselmann and McCormick 1995, Rommel, *et al* 1996). However, the function of the exon 1 domain of the BCR protein is unknown, whereas the BCR-ABL fusion protein requires this domain to activate the ABL component (Pendergast, *et al* 1991).

BCR exons 3-10 have regions of homology with Rho guanine-nucleotide exchange factors (Rho-GEF). This domain serves as a GDP-GTP exchange factor (GEF) for the small GTP-binding proteins, CDC42, Rho, Rac1, and Rac2 (Boguski and McCormick 1993, Cerione and Zheng 1996). These small molecules are associated with the formation of stress fibre, focal adhesions and membrane ruffling (Bussey 1996, Chuang, *et al* 1995, Quilliam, *et al* 1995). They also activate the mitogen activated protein kinase (MAPK)/ Jun-kinase (JNK) pathways as Ras does (Minden, *et al* 1995).

The COOH-terminal of BCR contains GTP-ase activating function for Rac (Rac-GAP) which regulates NADPH-oxidase function in phagocytes and induces membrane ruffling (Diekmann, *et al* 1991, Ridley, *et al* 1992, Voncken, *et al* 1995). Interestingly, both Rac-GAP and Rho-GEF domains can bind the small molecule Rac and related proteins in a

non-competitive manner suggesting they are important in stimulation of the members of the family of small GTP-binding proteins to help the activation of cell signalling and adhesion (Quilliam, *et al* 1995).

1.4.3. *More studies on the function of normal BCR and ABL*

More studies were done to search for other possible effects of tyrosine phosphorylation of BCR, particularly in the first exon sequences. The clue to study the first exon of BCR is because P190 BCR/ABL contains only BCR exon 1 but not other BCR exons and it was concluded that the major sites of BCR/ABL autophosphorylation were present in sequences encoded by the first exon of BCR (Liu, *et al* 1993). The work of Liu *et al* in 1996 surprisingly showed that the tyrosine phosphorylation of the BCR protein by the BCR/ABL protein strongly reduced its serine/threonine kinase activity (Liu, *et al* 1996b). The first exon of BCR which contains BCR 1-413, when compared to wild-type BCR/ABL is found that tyrosine residues Y283, Y328 and Y360 are sites of tyrosine phosphorylation resulting from BCR/ABL autophosphorylation apart from Y177 (Liu, *et al* 1996b, Pendergast, *et al* 1993b, Puil, *et al* 1994, Wu, *et al* 1998b). The further experiments were performed to test whether any of these tyrosine residues were involved in inhibiting the BCR serine/threonine kinase and found that the hydroxyl groups of tyrosine Y328 and 360 are critically involved in regulating BCR serine/threonine kinase activity (Wu, *et al* 1998b). The mutation of Y328 and Y360 to phenylalanine also showed the strong inhibition of the activities of the BCR protein (p160 BCR) (Wu, *et al* 1998b).

Other sites on the first exon of BCR have been discovered to be involved in supporting the BCR serine/threonine kinase activity and inhibit the tyrosine kinase function of c-ABL i.e. BCR 64-413 and phosphoserine 354 (S354) (Hawk, *et al* 2002). The co-expression of BCR 64-413 and over-expressed c-ABL revealed a reduction of tyrosine phosphorylation of c-ABL (Hawk, *et al* 2002). This may suggest the role of normal cytoplasmic c-ABL is blunted by the neutralization of normal BCR. Interestingly, the further studies have been done to determine the result between the expression of BCR 64-413 and BCR/ABL. The infection of cell lines with adenovirus encoding BCR 64-413 inhibited cell growth of K562 cells but not the pre-B cell lacking BCR/ABL, and BCR 64-413 expression induced high level of apoptosis in primary CML cells but not normal marrow cells (Wang, *et al* 2001).

These studies showed that normal BCR and c-ABL in cytoplasm had little role on the activities of normal marrow cells. BCR 64-413 antagonized the oncogenic effects of BCR/ABL but had no toxicity on haematopoietic cells lacking BCR/ABL. The studies on BCR above are compatible with the BCR-null mice experiment in which the mice developed normally and were fertile. In addition, haemopoiesis in the mice had no obvious defects. However, lack of BCR leads to deregulated superoxide production in neutrophils which is due to the lack of the Rac-GAP domain (Voncken, *et al* 1995). Similar to ABL, BCR-heterozygous mice do not have abnormalities suggesting that loss of a single allele of either BCR or ABL in CML does not lead to other defects.

In addition, full length of BCR protein was shown to reduce the level of phosphotyrosine BCR and increase phosphoserine BCR within the pool of total BCR dramatically in RAT-1 fibroblast cells transformed by BCR/ABL (Lin, *et al* 2001). This effect was not seen in the K562 cells and the induction of BCR expression in K562 caused an increase in cell differentiation (Wu, *et al* 1999).

As above, the BCR 64-413 is inhibitory to BCR/ABL, causing growth inhibition and cell apoptosis. In contrast, the S354 A mutant within BCR 64-413 had little effect on BCR/ABL positive cells (Hawk, *et al* 2002). The mechanism by which BCR 64-413 blocks the effects of c-ABL oncoprotein has been showed by Pendergast *et al.* that the serine-riched portion of BCR binds firmly to the SH2 domain of c-ABL and was not dependent on the presence of phosphotyrosine sequences (Pendergast, *et al* 1991). The A and B serine-riched boxes were identified within this region and the most effective binding was found in the B box which included S354 (Hawk, *et al* 2002, Lin, *et al* 2001, Liu, *et al* 1996a). This is surprisingly proven by the mutant S354 A of BCR 64-413 having no detectable ABL-SH2 binding ability (Hawk, *et al* 2002, Lin, *et al* 2001, Liu, *et al* 1996a). This suggested that the binding of phosphoserine BCR to ABL-SH2 domain perturbs the structure of SH2 domain and interferes with the function of ABL tyrosine kinase.

Y177, within another tyrosine kinase domain in the first exon of BCR, has been studied in mouse transplant models. These studies showed that the Y177 mutant of BCR/ABL is deficient in inducing myeloid leukemia (He, *et al* 2002, Zhang, *et al* 2001). This suggested

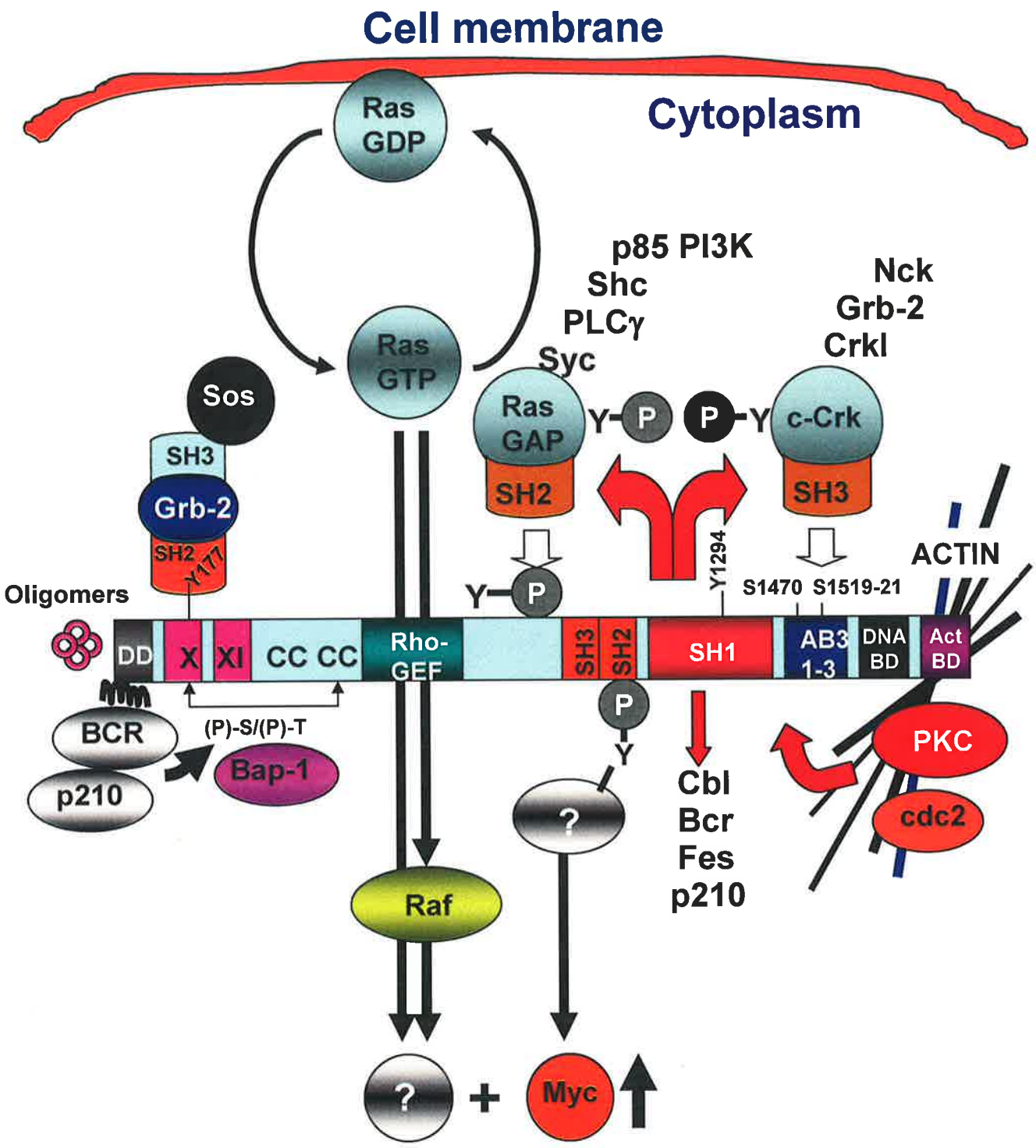
a role for Y177 in creating the oncogenic phenotype by the BCR/ABL oncoprotein (He, *et al* 2002, Zhang, *et al* 2001).

1.4.4. *The BCR-ABL fusion protein or $p210^{BCR-ABL}$ protein*

What is changed after the translocation event between chromosome 9q34 and chromosome 22q11 is that the BCR-ABL fusion gene is found at chromosome 22q with BCR sequences (chr.22q34) fused to ABL exon a2 (chr.9q11), and thus shortens chromosome 22 - the Philadelphia chromosome (Figure 1.1). The encoded protein $p210^{BCR-ABL}$ by this fusion gene deletes exon 1 of the normal c-ABL which is the myristoylation site of 1b isoform c-ABL, and also inhibits the function of SH3 domain to open the 'Fold-back' structure. This causes the SH1 domain of the ABL portion to have an uncontrolled tyrosine-kinase activity due to phosphorylation of SH1 itself. The uncontrolled tyrosine kinase activity not only results from the uncapped SH3 domain but is also believed that the first 63 a.a. of the BCR portion, containing the coiled-coil domain, allows tetramerisation (McWhirter, *et al* 1993, McWhirter and Wang 1991), which is necessary to activate the tyrosine kinase function, as well as to activate the F-actin binding function of ABL causing BCR-ABL protein to be cytoplasmically located (McWhirter and Wang 1993, Van Etten, *et al* 1989). The mutation of the coiled-coil motif domain which falls into the BCR amino acid 1-63 in a murine model failed to induce the murine CML disease and this effect was thought to have resulted from the moderate depression of tyrosine kinase activity caused by the inability of this mutant to oligomerize (Wertheim, *et al* 2002). The tyrosine Y177 in the BCR portion of the fusion protein is phosphorylated by the ABL tyrosine kinase and then serves as the docking site for the SH2 domain of the adapter protein Grb-2 (Pendergast, *et al* 1993b), whereas the SH3 domain of Grb-2 is the binding site for mSos-1, a guanine nucleotide exchange factor (Puil, *et al* 1994, Saxton, *et al* 1994) (Figure 1.5). The mSos-1 has the GTP-ase activity for Ras by promoting the conversion of GDP-bound Ras to GTP-bound Ras (Figure 1.5). The GTP-bound Ras then activates the Ras signal transduction (Tauchi, *et al* 1994). Interestingly, the mutation of Tyr^{177} or Y177 region dramatically reduces the ability of BCR-ABL to transform primary haemopoietic cells and rodent fibroblasts (Pendergast, *et al* 1993b) suggesting the activation of Ras is critical.

Figure 1.5. The function of the fusion protein $p210^{BCR/ABL}$

This diagram is a summary of the functions of BCR/ABL. The BCR/ABL forms homo-oligomers or hetero-oligomers with BCR serine threonine kinase through an N-terminal dimerisation domain (DD). This protein is localised only in the cytoplasm in contrast to the normal c-ABL which shuttles between nucleus and cytoplasm. The autophosphorylation of BCR/ABL creates the binding site or docking site for many protein substrates to bind with the BCR/ABL and causes the phosphorylation of those protein substrates.



Adapted from Laneuville *et al.* 1995

Figure 1.5

The phosphoserine/threonine ((p)-S/(p)-T) rich SH2 binding domain located between amino acids 192 and 413 of BCR portion of BCR/ABL binds to Bap-1 (Maru and Witte 1991, Pendergast, *et al* 1991, Reuther, *et al* 1994) which is a member of 14-3-3 family. This may activate the tyrosine kinase activity of ABL portion (Pendergast, *et al* 1991) by inhibition of the serine/threonine kinase, which normally inhibits the ABL tyrosine kinase activity. Owing to the ability of other members of 14-3-3 family to stimulate c-Raf (Braselmann and McCormick 1995, Papin, *et al* 1996, Rommel, *et al* 1996) and the binding of Bap-1 to BCR in close proximity to Grb-2 on BCR (Pendergast, *et al* 1993b), it is possible that the Bap-1 may activate Raf and consequently result in BCR-ABL-mediated signaling through the MAPK and/or JNK pathways (Goga, *et al* 1995a, Goga, *et al* 1995b, Skorski, *et al* 1995). In addition, a previous report showed that 14-3-3 molecules may associate with integrin adhesion receptors (Du, *et al* 1996), therefore BCR-associated Bap-1 in CML cells may cause the adhesion defect seen in CML cells as well. The Rho-GEF or DBL-like domain is important for the regulation of superoxide production in normal nutrophils but its function in CML cells is still unknown. It may have an interaction with the Rac-GAP domain to gain the full activation of the small GTP-binding protein. The function of the Rho-GEF domain may not be active owing to the deletion of Rac-GAP domain in many types of BCR-ABL fusion protein.

The study of v-ABL suggests that the deletion of ABL SH3 domain is essential for the autophosphorylation of ABL tyrosine kinase domain (Mayer and Baltimore 1994). However, this domain is still intact in the BCR-ABL fusion protein suggesting that the SH3 domain in the BCR-ABL fusion protein may have only minimal inhibitory effects on ABL tyrosine kinase function and supports the unfolded 'Fold-back'structure which may play a role in the activation of the ABL tyrosine kinase domain. Three other regions on ABL portion are required for CML transformation. Firstly, the F-actin and G-actin domains at the COOH-terminal of the BCR-ABL molecule have been shown to be critical for the cell-transformation, because the transforming ability of BCR-ABL is significantly reduced if these domains are deleted (McWhirter, *et al* 1993, McWhirter and Wang 1991, Renshaw, *et al* 1995, Van Etten, *et al* 1989). In addition, the focal adhesion of BCR-ABL protein to F-actin and G-actin disrupts the normal binding of integrins to integrin receptors and lateral association of integrin receptors which is important for signal transferred

through the cytoskeleton from cytokine or integrin receptors in the plasma membrane (Lewis, *et al* 1996). This leads to the premature migration of CML progenitors from the bone marrow micro-environment (Gordon, *et al* 1987, Verfaillie, *et al* 1997) through these integrin based mechanisms. The focal adhesion of BCR-ABL protein to F-actin or G-actin is also a factor keeping BCR-ABL localised in the cytoplasm where the constitutively activated tyrosine kinase can interact with the key proliferation and survival signaling molecules easily and effectively (McWhirter and Wang 1993, Van Etten, *et al* 1989). The second and third regions are SH1 and SH2. The SH1 tyrosine kinase domain of the BCR-ABL is responsible for phosphorylation of a number of tyrosines in BCR and ABL portions of BCR-ABL fusion protein itself (Pendergast, *et al* 1993a) as well as a large number of adaptor and signal proteins. These include c-Cbl (Andoniou, *et al* 1994, de Jong, *et al* 1995, Sattler, *et al* 1996) and Sch (Goga, *et al* 1995b, Tauchi, *et al* 1994) both of which can bind to the SH2 site of ABL upstream of tyrosine kinase domain, and Crkl (de Jong, *et al* 1995, Oda, *et al* 1994, Sattler, *et al* 1996) which can bind to a proline-rich motif downstream from the tyrosine kinase domain. As the binding of these substrates is close to the SH1 tyrosine kinase domain, this allows these substrates to be phosphorylated and creates the docking sites for cytoplasmic adaptor molecules (Harrison-Findik, *et al* 1995, Sattler, *et al* 1996). The substrates and adaptor proteins that are activated or bind to c-ABL and BCR-ABL are shown in Table 1.1. Overexpression of Myc has been found in many human malignancies, although the target genes are unknown. In BCR-ABL driven cells, activation of Myc depends on the SH2 domain on the ABL portion of BCR-ABL(Sawyers, *et al* 1992). However, the pathway linking Myc to SH2 domain of BCR-ABL is still unknown. The studies on v-ABL-transformed cells suggest the signal transduced through Ras/Raf, cyclin-dependent kinases (cdks), and E2F transcription factors can activate the Myc promotor (Zou, *et al* 1997).

1.5. ***Cellular signaling pathways relating to the BCR/ABL protein***

It is quite clear that BCR/ABL protein is necessary for the proliferation of CML cells. The proliferation activation occurs through a number of signal transduction proteins such as Ras and PI-3K pathways. In turn these pathways are at least in part mediated by adaptor proteins such as growth receptor binding protein-2 (Grb-2), the Crk like proteins (Crkl) and

Shc. The adaptor proteins function as the link between BCR/ABL and signaling proteins, and help the BCR/ABL to activate these signaling proteins (McCubrey, *et al* 2000).

1.5.1. *Ras pathways*

The Ras proteins are classified within the large Ras superfamily of monomeric GTPases. Other proteins in this superfamily include Rho and Rac proteins which are involved in relaying signals from cell surface receptors to the actin cytoskeleton, and Rab which is involved in the trafficking of intracellular vesicles (Beaupre and Kurzrock 1999). The link between Ras and BCR/ABL has been defined through the phosphorylation of Y177 on the BCR portion of BCR/ABL which provides the docking site for the adapter molecule Grb-2 (Pendergast, *et al* 1993b). Grb-2 then binds to m-SOS which converts the GDP-bound Ras to GTP-bound Ras. This stabilizes Ras in the GTP-bound form (Pendergast, *et al* 1993b).

In the absence of Grb-2, other adaptor molecules such as Shc and Crkl provide alternative activators of Ras for haemopoietic transformation (Nichols, *et al* 1994, ten Hoeve, *et al* 1994). However, Ras activation by Crkl has only been demonstrated in fibroblasts (Senechal, *et al* 1996), and the binding of Crkl to BCR-ABL is not essential for the transformation of myeloid cells (Heaney, *et al* 1997). Unlike acute myeloid leukaemia or other malignant tumors, Ras is unlikely to have activating mutations even in blastic phase of CML. This leads to the understanding that Ras is essential for the pathogenesis of Ph-positive leukaemias (Watzinger, *et al* 1994). Normally, Ras will be activated through cytokine receptors such as IL-3, and subsequently leads to the recruitment of serine/threonine kinase Raf to the cell membrane (Marais, *et al* 1995). The association of Ras to Raf then leads to stimulate a signaling cascade through the serine-threonine kinases Mek1/Mek2 and the two MAPKs (also called Erk1 or Erk44, and Erk2 or Erk42) which in turn move to the nucleus and then activate the nuclear gene transcription factors (such as Elk-1) of cells (Cahill, *et al* 1996, Crews, *et al* 1992, Dhanasekaran and Premkumar Reddy 1998, Marais, *et al* 1993). Some data have shown that this pathway is only found in v-ABL but not BCR-ABL transformed cells (Kabarowski, *et al* 1994), therefore the activation of MAPK pathway is still controversial in BCR/ABL transformed cells. In BCR/ABL cells, Ras may be relayed through the GTP-GDP exchange factor Rac (Skorski, *et al* 1998) to Gckr (Germinal center kinase related) (Shi, *et al* 1999) and then to Jnk/Sapk, because the

activation of Jnk/Sapk pathway by BCR-ABL has been reported and is claimed that to be essential for malignant transformation (Raitano, *et al* 1995). The Jnk/Sapk pathway is involved in the regulation of cell cycle and apoptosis (Fuchs, *et al* 1998, Nebreda and Gavin 1999). In addition, the Ras/Raf pathway may modulate the cell cycle by the induction of expression of cyclin D1, repression of expression of $p27^{kip1}$ and the induction of expression of c-Myc and the cyclin D kinase inhibitor $p21^{WAF1/cip-1}$ (Kerkhoff and Rapp 1998).

Both BCR/ABL and a variety of growth factors lead to the phosphorylation of RAS pathway-associated proteins (Shc, SHIP, Erk-2, RAS-GAP), paxillin, JAK/STAT pathway proteins (STAT1, STAT5, JAK1 and 2, and TYK2), the phosphatases SHP1 and 2, CRKL, and c-Cbl, the β_C of certain growth factor receptors (e.g. IL-3), c-KIT and a number of other tyrosine kinases and substrates (Sattler and Salgia 1997). CML expansion may relate to some cytokine receptors and may lead to the primary CML surviving specific ABL kinase blockade. BCR/ABL protein is believed to transduce signals through growth factor receptors because the association of β_C of the IL-3 receptor (Wilson-Rawls, *et al* 1996)and the Kit receptor (Hallek, *et al* 1996)with the BCR-ABL protein has been observed. Another example is the observation of the link between BCR/ABL and SHIP. SHIP has been demonstrated to reduce its expression directly by the expression of BCR/ABL and this reduction could be reversed by the imatinib treatment (Sattler, *et al* 1999).

1.5.2. *Phosphotidylinositol 3'kinase pathway (PI3-K)*

PI3K is a family of proteins that when activated will transfer the G-phosphate of ATP to the D3 position of phosphoinositides and phosphorylates the substrates downstream such as Akt. This protein family is devided into three classes. Class I is regulated by growth factor receptor activation and consists of an approximately 110 kDa subunit containing both lipid and serine/threonine kinase activity and a regulatory subunit of 50-110 kDa (Hawkins, *et al* 1997, Stephens, *et al* 1993) in which the p85 subunit contains SH2 and SH3 domains acting as an adaptor protein, allowing the p110 catalytic subunit to interact with receptor and non-receptor protein tyrosine kinases (PTK's), leading to PI-3 kinase activation (Margolis 1992). Class I is involved in for the binding of phosphoinositide-(4,5) diphosphate (Hawkins, *et al* 1997, Stephens, *et al* 1993). Class II PI3K which is necessary

for phospholipid binding is a single peptide of 170-210 kDa with a carboxyterminal C2 domain (MacDougall, *et al* 1995) (Misawa, *et al* 1998). Class III is associated with *Saccharomyces cerevisiae* vacuolar protein sorting mutant (Schu, *et al* 1993). The most interesting PI3K class involving in the signalling of haemopoietic cells is the class I - PI3K. Activation of PI3K needs the translocation to the plasma membrane and association with an activated receptor tyrosine kinase or its substrates (van der Geer, *et al* 1994, Wymann and Pirola 1998). Its plasma membrane localization is activated by IL-3 receptor, Ras or the proline-rich regions of Shc, Lyn, Fyn, Grb2, v-Src, ABL, Lck, Cbl, or dynamin (Kapeller, *et al* 1994, Mak, *et al* 1996, Pleiman, *et al* 1994, Wang, *et al* 1995) (Harrison-Findik, *et al* 2001, Hu, *et al* 1993, Soltoff and Cantley 1996, Stoyanova, *et al* 1997) (Dombrosky-Ferlan and Corey 1997, Hunter, *et al* 1997). These interactions activate the phospholipid products i.e. phosphoinositide-3 phosphate and/or phosphoinisitide-3,4,5-triphosphate, both of which further the activation of downstream targets including PDK, AKT and PKC (Nakanishi, *et al* 1993, Palmer, *et al* 1995, Toker, *et al* 1994). The regulatory domain or p85 domain of PI-3K has been showed to co-immunoprecipetate with BCR-ABL in cell lines (Gotoh, *et al* 1994) and primary CML cells (Skorski, *et al* 1995). Skorski *et al.* have shown that the proliferation of Ph-positive cell lines and colony formation of CML primary cells were inhibited by using specific antisense oligonucleotides that down-regulate the p85 subunit and by wortmannin, a potent inhibitor of the p110 catalytic subunit (Skorski, *et al* 1995). This suggests the PI3-K is one of the effectors of BCR/ABL tyrosine kinase in CML cells.

AKT protein is one of the downstream targets of PI3K activation which contains three domains: the PH (the pleckstrin homology domain), the serine/threonine kinase and the regulatory domains (Nicholson and Anderson 2002). The translocation of activated PI3K to the membrane causes PIP3 (phosphoinositide-3 phosphate) binding to the AKT-PH domain (Nicholson and Anderson 2002). Then PI3K phosphorylates phosphoinositides causing the translocation and activation of PDKs (phosphotidylinositol dependent kinases). PDK1 first phosphorylates and activates AKT on Thr 308, and then PDK2, which although not fully characterised, is thought to phosphorylate Ser 473 of Akt (Nicholson and Anderson 2002, Stephens, *et al* 1998). In BCR/ABL expressing cells, the activated AKT phosphorylates the Forkhead transcription factor, FKHRL is then bound by 14-3-3

inhibiting the its pro-apoptotic activation (Brunet, *et al* 1999, Komatsu, *et al* 2003). The net result of the activation through this pathway causes the proteasome-mediated degradation of the key cell cycle inhibitor $p27^{Kip1}$ (Gesbert, *et al* 2000, Jonuleit, *et al* 2000). The suppression of $p27^{Kip1}$ which is found in CML progenitor cells causes accelerated entry into the S-phase of cell cycle (Jonuleit, *et al* 2000). The activation of AKT also has anti-apoptotic activity (Franke, *et al* 1997). The key substrate of the anti-apoptosis through AKT activation is Bad (del Peso, *et al* 1997). Bad is a mitochondria protein which can promote apoptosis by binding and inhibiting pro-survival proteins in mitochondria such as Bcl-2 and Bcl-$_{XL}$. When Bad is phosphorylated specifically by PI-3K/AKT pathways, it translocates to the cytoplasm where it then is sequestered by the 14-3-3 protein, blocking the migration of Bad from cytoplasm to mitochondria (Brunet, *et al* 1999, Fu, *et al* 2000, Zha, *et al* 1996). A recent report showed that AKT is in the downstream cascade of the IL-3 receptor (del Peso, *et al* 1997). SHIP-1 and SHIP-2, the 2-inositol-phosphatases which are activated by cytokines of the IL-3 family and involved in the PI3-K/AKT pathway are also activated by BCR/ABL (Lioubin, *et al* 1996, Wisniewski, *et al* 1999). The details about SHIP-1 and SHIP-2 will be explained later. Together, this indicates that BCR/ABL might mimic the physiologic IL-3 survival signal in a PI3-K dependent manner. Some studies have shown that despite complete dephosphorylation of Bad, a fraction of cells survived treatment (Neshat, *et al* 2000), suggesting cell survival by Bad-independent pathways.

Recently there have been reports about the negative regulation of PI3-K. PTEN (phosphatase and tesin homolog deleted on chromosome 10), which is considered a tumor suppressor gene, is a dual specific lipid and protein phosphatase that removes 3'-phosphatase from the PI3-K lipid product phosphotidylinositol $(3,4,5)P_3$ to be phosphotidylinositol $(4,5)P_2$, which prevents AKT activation (Leslie, *et al* 2000, Rohrschneider, *et al* 2000, Wu, *et al* 1998a). Like PTEN, SHIP-1 and SHIP-2, two other phosphatases, were shown to remove the 5-phosphatase from the phosphotidylinositol $(3,4,5)P_3$ to produce phosphotidylinositol (3,4) P_2 (Muraille, *et al* 1999, Taylor, *et al* 2000). The PTEN and SHIP-1 have been found to be mutated and ineffective in some human cancer and leukaemias (Luo, *et al* 2003, Muraille, *et al* 1999).

1.5.3. *C-Cbl and Crkl pathways*

C-Cbl (Castitas B lineage lymphoma) is a 120 kDa proto-oncogene product which is tyrosine phosphorylated in v-ABL and BCR/ABL transformed cells (Andoniou, *et al* 1994, Gotoh and Broxmeyer 1997). In CML cells, C-Cbl associates with multiple proteins including paxillin and talin (Salgia, *et al* 1996) which are involved in focal adhesion. C-Cbl also binds the p85 subunit of PI3-K (Sattler, *et al* 1996). It is thought from this data that C-Cbl is downstream of BCR/ABL but upstream of PI3-K. In addition, C-Cbl interacts with the adaptor protein Crkl in CML cells (de Jong, *et al* 1995, Sattler, *et al* 1996).

Apart from C-Cbl, Crkl binds a number of proteins including p130 CAS, paxillin, c-ABL, BCR/ABL, SOS, and PI3-K (Sattler and Salgia 1997) and behaves as a bridge to recruit other substrate proteins such as SOS and PI3-K close to BCR/ABL and consequently those substrates are phosphorylated by BCR/ABL.

1.5.4. *Myc pathway*

The Myc pathway is classified as oncogenic due to its overexpression in many kinds of human malignancies. Interestingly, the SH2 domain and the C-terminus of BCR/ABL protein are required for full activation of Myc even though no link between Myc and the SH2 and C-terminus domain of BCR/ABL is known (Afar, *et al* 1994, Okuda, *et al* 1998). Studies on v-ABL suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinase (cdks), and E2F transcription factors may activate the Myc promotor (Stewart, *et al* 1995, Zou, *et al* 1997). Recently, JAK2 has been shown to be involved in Myc induction by BCR/ABL, by the induction of Myc mRNA and by stabilization of the protein (Xie, *et al* 2001). This might be further evidence for the cross talk between BCR/ABL, Myc, and the IL-3 receptor. Myc may provide dual activity inducing both proliferation and apoptosis for the normal cells depending on cellular context (Bissonnette, *et al* 1992, Evan, *et al* 1992). Therefore, other mechanisms may counterbalance the apoptosis side of the dual function of Myc in the activation through BCR/ABL in CML cells such as Ras and PI3-K pathways.

1.5.5. *Jak/STAT*

It was first reported in the study of v-ABL-transformed B cells that the activated ABL could stmulate the interaction between Jak/STAT pathway (Danial, *et al* 1995). However, the BCR/ABL has a pleiotropic physiology on the activation of this pathway by bypassing the normal physiologic Jak2/STAT5 activation sequence (Nosaka, *et al* 1999). The effect of STAT5 was found to inhibit apoptosis of the BCR/ABL transformed cells by involving the transcriptional activation of BCL-XL (Horita, *et al* 2000, Sillaber, *et al* 2000). The physiologic activity of this pathway found in the BCR/ABL negative cells was activated through cytokine receptors including IL-3 family receptors which involve the normal sequence of Jak/STAT activation (Darnell, *et al* 1994).

There are 4 mammalian Jaks including Jak1, Jak2, Jak3 and Tyk2 that interplay with the cell surface cytokine receptors, particularly the non-tyrosine kinase receptors (Ihle 1996, Taniguchi 1995). Only a few tyrosine kinases receptor activate Jak, such as stem cell factor and M-CSF, but the role of Jaks on tyrosine kinase receptors are not clearly understood (Deberry, *et al* 1997, Novak, *et al* 1995). Jak is a tyrosine kinase molecule as well. It behaves to promote cell signaling through the non-tyrosine kinase. On the other hand, non-tyrosine kinase receptors require the tyrosine kinase molecules like Jak to help activation or initiation of activation of their substrates. The normal physiologic sequence of STAT activation through the cytokine receptors first needs activation of Jak.

STAT is a cytoplasmic protein transcription factor which sits latent in the cytoplasm. This protein becomes activated when the recruitment or activation of cytokine receptors occurrs. There are seven isoforms of STATs that have been identified so far includimg STAT-1, -2, -3, -4, -5a, -5b, and STAT-6 ranging from molecular weight 75 to 95 kDa (748-851 aa.) (Steelman, *et al* 2004). Phosphorylation of STAT5 is found in the activation of cytokine receptors of the IL-3 superfamily and also through the activation by BCR/ABL. The activation of STAST5 cause the dimerisation of this protein which will promote movement of STAT to the nucleus, as well as DNA binding and activation of transcription (Steelman, *et al* 2004).

1.6. *Haemopoietic cytokines of the IL-3 family involved in signaling through BCR/ABL*

During chronic phase, CML progenitors are still depend on external growth factors for their survival and proliferation (Amos, *et al* 1995). In addition, recent reports showed that BCR/ABL induced IL-3 and G-CSF and also cytokine receptors in the early progenitor cells such as the oncosatin M β receptor (Deininger, *et al* 2000b). Therefore, this may reflect that the chronic phase CML progenitors may require cytokines for disease expansion even though no strong evidence for this has yet been demonstrated.

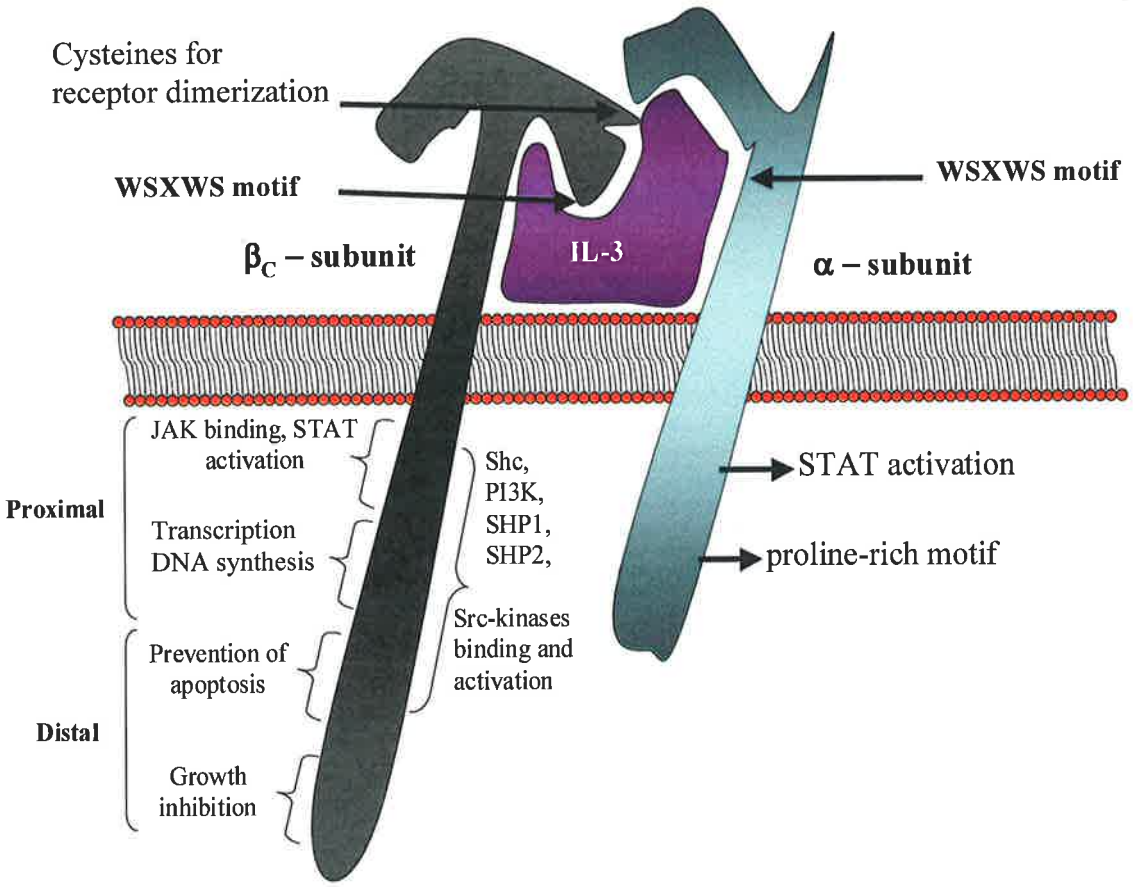
The haemopoietic process is regulated by a number of soluble factors called cytokines. These molecules bind to their corresponding receptors and stimulate intracellular signal transduction. The functional characteristics of cytokines not only are pleiotropic but also redundant. A single cytokine may mediate the growth and differentiation of many kinds of cell lineages, and in addition could play role in concert with other stimulators or cytokines on the same cell lineage for particular cell functions. Cytokine receptor families have been sub-divided according to the characteristic structural motifs in their extracellular domains (Hunter 1993, Kishimoto, *et al* 1994, Stahl and Yancopoulos 1993, Taniguchi 1995). The main sub-groups are the PTK (protein tyrosine kinase) receptor family, the gp-130 family, the IL-2 receptor family, the growth hormone family, the interferon family and the gp-140 family (Hunter 1993, Kishimoto, *et al* 1994, Stahl and Yancopoulos 1993, Taniguchi 1995). The PTK receptor family possesses a tyrosine kinase domain hinge which will recruit and transfer a phosphate group to downstream substrate. The remaining families are non- PTK families which create the docking sites for intracellular tyrosine kinases to bind after the receptor conformation is altered by forming a high affinity complex with their cognate ligands.

1.6.1. *IL-3 receptor super family*

The IL-3 receptor superfamily which includes GM-CSF, IL-3 and IL-5 is classified within the gp-140 family. GM-CSF, IL-3 and IL-5 are haemopoietic cytokines involved in the activation of myeloid cells and their precursors through cytokine cell surface receptors which are composed of β_C (common beta-chain) and individual specific α receptors, and share the β_C common receptor (Figure 1.6) (Bagley, *et al* 1997). The receptors for these cytokines are defined by the specific α receptors which have little role in direct signal

Figure 1.6. The IL-3 receptor superfamily

This diagram is the representative of the cytokine receptors in the IL-3 receptor superfamily including IL-3, IL-5 and GM-CSF. Each receptor comprises a unique ligand binding α-subunit that is not shared by other members in this family. The signal transduction mostly occurs through the common β –receptor which is shared by the receptor for IL-3, IL-5 and GM-CSF.



Adapted from EP Reddy *et al.* 2000

Figure 1.6

transduction (Bagley, *et al* 1997, Woodcock, *et al* 1999). The β_C is important for the recruitment and then activation of tyrosine kinase molecules in the cytoplasm, which is important for the downstream signal transduction (Bagley, *et al* 1997, Woodcock, *et al* 1999). Now we are going to use the IL-3 receptor as a model to explain how signals are transduced by the receptors in this family.

The specific α chain can only bind with its specific cognate cytokine ligand with low affinity with $K_d \geq 0.5$ nM and is inadequate for full receptor activation. The β_C subunit alone is unable to bind any of the cytokines. However, in the presence of α chain, the β_C can show high affinity for the ligand: α chain complex with a K_d of ~100 pM and then mediate receptor activation (Woodcock, *et al* 1999). Both subunits contain the extracellular ligand binding domain, the transmembrane and the intracellular signaling domains. To activate the receptors, first ligand has to bind with the α chain, and then β_C subunit will be recruited to form a high affinity receptor complex to the ligand:α chain (Woodcock, *et al* 1999). Otherwise the ligand:α chain complex will dissociate rapidly (Woodcock, *et al* 1999). Interestingly, on cells which have appropriate α chains and limiting number of β_C chains, GM-CSF, IL-3 and IL-5 can cross-compete for each other and GM-CSF shows greatest ability to recruit β_C, followed by IL-3 and IL-5 (Lopez, *et al* 1989, Lopez, *et al* 1992, Lopez, *et al* 1991). The ligand binding domains of α subunits differ in their specificity, varying among GM-CSF, IL-3 and IL-5 receptors. Seemingly, α subunit is not essential for signal transduction because the cytoplasmic domain of the α subunit is very short and has no detectable catalytic domains (Matsuguchi, *et al* 1997). Although the cytoplasmic domain of the α subunit is unable to signal in the absence of the β_C subunit (Robb, *et al* 1995), the cytoplasmic domains are absolutely required for receptor activation (Barry, *et al* 1997, Ronco, *et al* 1994). Apart from the ligand binding function, the α intracellular domain is found to be necessary for the activation of STAT 5 (Doyle and Gasson 1998, Matsuda and Hirano 1994), and mediates the transcriptional activation of c-fos, c-jun, c-Raf and p70S6K (Itoh, *et al* 1996, Tian, *et al* 1996), (Watanabe, *et al* 1995).

The cytoplasmic domain of the β_C contains a proximal portion spanning amino acids 1-589 that includes the Box 1 region, and the distal portion from a.a. 589-881 (Sato, *et al* 1993, Tian, *et al* 1996),(Inhorn, *et al* 1995. The proximal region is important and sufficient for

DNA synthesis, required for the induction of proliferation associated genes such as c-Myc, pim-1 and oncostatin M, and cytokine induced dimerization which was found to bind to several signal transducing proteins i.e. JAKs, STAT, c-Src, PI3K, and Vav {Quelle, 1994 #23, Jaster, *et al* 1997, Matsuguchi, *et al* 1995, Rao and Mufson 1995, Yoshimura 1998, Yoshimura, *et al* 1995). The distal region required for the growth inhibition which can be induced by some cytokines, is necessary for the promotion of viability of haemopoietic cells, and is found to interact with other signal proteins i.e. Shc and SHP and mediate transcriptional activation of c-fos and c-jun as well as c-Raf and p70S6K (Inhorn, *et al* 1995).

The β_C subunits lack intrinsic kinase activity. The process or cascade of signal transduction through β_C subunit requires conformational changes in the cytoplasmic domains. Hetero-dimerization between the α and β_C subunit promotes the binding and activation of the downstream targets including JAK, STAT and PI3-K and other shuttling molecules such as Shc by transducing the signal through Grb2 and SOS to the Ras/Raf/MEK/ERK cascade (Blalock, *et al* 1999, Miyajima, *et al* 1993).

1.6.2. *GM-CSF and its receptor*

GM-CSF is located on the long arm of chromosome 5 (5q21-q32) about 9 kilobase-pairs downstream from the hIL-3 gene (van Leeuwen, *et al* 1989, Yang, *et al* 1988). There are a number of cytokines and cytokine receptors encoded by chromosome 5q. Those include hIL-4, IL-5, M-CSF, c-fms (the M-CSF receptor) and an early growth response gene (EGR-1). The deletion of this chromosome or some part of chromosome 5q is found in some diseases i.e. therapy-related myelodysplastic syndrome, acute leukaemia and the 5q-syndrome (Le Beau, *et al* 1986, Nimer and Golde 1987, van Leeuwen, *et al* 1989), (Le Beau, *et al* 1989).

With molecular weight of 22,000, mGM-CSF was first described and purified from mouse-lung conditioned medium in 1977 by Burgess *et al.* (Burgess, *et al* 1977) and the human form hGM-CSF was purified from medium conditioned by the HTLV-II-infected T lymphoblast cell line, Mo (Gasson, *et al* 1984, Golde, *et al* 1978). This cytokine is synthesized by many cell types in response to specific activating signals (Table 1.2). It is

produced and functions in an autocrine or paracrine fashion. The production of GM-CSF is controlled by a combination of both transcriptional and post-transcriptional regulators. Its gene is found to have constitutive transcription in monocytes, endothelial cells and fibroblasts despite no detected mRNA accumulation (Koeffler, *et al* 1988, Thorens, *et al* 1987). The aberrant of accumulation of mRNA to detectable levels suggested its involvement in the pathogenesis of CML (Schuler and Cole 1988).

Interestingly, in addition to role in normal haemopoiesis, GM-CSF has been implicated in the pathogenesis of a number of leukemias. Blast cells from acute myeloid leukemia frequently produce and respond to this factor, reducing or abrogating their requirement for exogenous growth factors in an autocrine manner (Young and Griffin 1986, Young, *et al* 1987). The constitutive transcription of GM-CSF in monocytes is also found in some types of myelodysplastic syndromes (Gualtieri, *et al* 1989) and ALL (Freedman, *et al* 1993). This leads to the examination of the relationship between GM-CSF and the CML transformation, because some clinical features of CML and myelodysplastic syndrome are similar, suggesting they share some common factors.

1.6.3. The importance of cytokine and cytokine receptors in haemopoietic disease and neoplasia

Normally, detectable levels of the cytokines of the IL-3 family cannot be observed in both normal bone marrow and CML bone marrow. This contributes to the hypothesis that the cells may produce cytokines locally in an autocrine/paracrine fashion. Holyoake *et al.* (Holyoake, *et al* 2001) has shown that the rare subset of quiescent leukaemic $CD34^+$ cells derived from CML patients in chronic phase were able to generate clones in the absence of growth factors, and few quiescent CML cells produced detectable levels of either growth factor transcript (IL-3 and G-CSF m-RNA) in the presence of BCR/ABL m-RNA. However, in a previous study the evaluation of $CD34^+$ BCR/ABL^+ cells they found 80% of CML $CD34^+$ cells contain detectable level of IL-3 mRNA and 50% have G-CSF (Jiang, *et al* 1999). The non-$CD34^+$ cells which were BCR/ABL^- did not produce detectable mRNA transcript of any growth factors (Jiang, *et al* 1999). Although the quiescent cells in this population are only rare, the ability to generate clones and colonies does not mean this is the effect of BCR/ABL only. They may survive and grow by the supporting substances

produced and acting locally, not by the quiescent cells but by committed cells, both progenitors and non-progenitors. Their later work has shown that by culturing patient cells in serum deprived medium for 4 days with or without growth factors and examining the rate of cell cycle entry from G_0 stage, the quiescent cells appeared activated and divided in both the presence and absence of growth factors (Holyoake, *et al* 2001). Interestingly, the IL-3 transcript was markedly increased in the quiescent cells in the absence of growth factors whereas no detectable IL-3 transcript was observed in those with added growth factors (Holyoake, *et al* 2001). This suggests BCR/ABL may be involved in the production of some growth factors by quiescent CML cells, which may promote cells entering to cell cycle and perhaps induce quiescent stem cells to become more mature committed stem cells. The committed stem cells as we know are more active in entering cell cycle than quiescent cells, and are more sensitive to some stimulators or cytokines than quiescent stem cells. A study demonstrated that CML progenitors have earlier maturation than comparable normal progenitors. That study showed that in the $CD34^+$ cells in CML cells compared with normal cells, the percent of HLA-DR^+ CML $CD34^+$ cells is much higher than normal HLA-DR^+ $CD34^+$ cells (Udomsakdi, *et al* 1992). The expression of cell surface antigen HLA-DR normally suggests early response to growth factors (Delforge, *et al* 1999, Kirk, *et al* 1995, Verfaillie 1992). In addition, the HLA-DR^- $CD34^+$ cells derived from CML patients in chronic phase are generally Ph^- and polyclonal. The HLA-DR^- CML $CD34^+$ cells are thought to be decreased or disappeared during the progression of disease(Dube, *et al* 1984, Udomsakdi, *et al* 1992). These HLA-DR^- cells are also very primitive cells because the quiescent CML cells which are non-cycling have been reported to express very low levels of HLA-DR compared to cycling $CD34^+$ cells(Holyoake, *et al* 2001). The conclusion that the decrease of the HLA-DR^- population in CML is due to the suppression of HLA-DR^- cells during disease progression might be in question. A more likely explanation may be that the HLA-DR^- cells become HLA-DR^+ cells by internal or external stimulation. These findings collectively suggest that the primitive CML cells which are mostly HLA-DR^+ are susceptible to the stimulation of some growth factors, particularly IL-3, and the primitive cells which are negative for HLA-DR may become positive for HLA-DR and then become susceptible to auotocrine/paracrine cytokines or exogenous cytokines. This suggestion is confirmed by a study by Jiang *et al.* (Jiang, *et al* 2002) showing that the generation of leukaemia was critically dependent on the persistence

of an autocrine IL-3 mechanism when the production of other growth factors were attenuated by short-term passage of BCR/ABL transduced cell in culture prior to the transplantation of reduced numbers of leukemic stem cells and were not useful to maintain the survival of stem cells in fluid culture. Therefore, they concluded the maintenance of leukemic stem cells required IL-3. Although there is evidence for the spontaneous growth of CML cells by activity of their BCR/ABL protein alone, in primary CML the BCR/ABL may not reduce the effect of growth factor receptors completely as has been found in the BCR/ABL cell lines.

1.7. Proposed studies in this thesis

1.7.1. *Cell growth driven by BCR/ABL is cytokine dependent*

Several studies relating to cytokines and BCR/ABL in CML have provided many interesting data. In the context of BCR/ABL-induced leukaemia, the BCR/ABL protein itself can induce the secretion of multiple cytokines such as IL-3, GM-CSF and G-CSF (Deininger, *et al* 2000a, Jiang, *et al* 1999, Verfaillie, *et al* 1997, Zhang and Ren 1998). A study using the myeloid cytokine-dependent cell FDCP-1 cell line showed that the expression of BCR/ABL induces secretion of IL-3 in an SH2 dependent fashion (Anderson and Mladenovic 1996, Hariharan, *et al* 1988). However, the growth and survival of IL-3 dependent cell lines with BCR/ABL does not involve an autocrine mechanism (Daley and Baltimore 1988, Hariharan, *et al* 1988, Ilaria and Van Etten 1995). In the study by Zhang and Ren in 1998, both BCR/ABL transfected and non-transfected myeloid cells were massively expanded in mice with the BCR/ABL-induced myeloproliferative disease (Zhang and Ren 1998). This bystander effect also suggests that the excess growth factors may be produced in the BCR/ABL mice and also introduces the controversial idea that the growth factors may play a role in autocrine/paracrine stimulation of growth of myeloid cells in CML. Not only IL-3 and GM-CSF levels were detected in serum of mice with BCR/ABL-induced CML-like disease, but increased serum levels of GM-CSF and increased gene expression of IL-3 in CML primitive hemopoietic progenitor cells have also been reported in CML patients (el-Ahmady, *et al* 1997, Jiang, *et al* 1999, Jonuleit, *et al* 1998).

An interesting point is if cytokine produced by BCR/ABL cells affect the bystander myeloid cells, why are most of the expanded cells in CML patients CML cells? CML cells of patients, in contrast to those of BCR/ABL engineered mice, may be more susceptible to the action of cytokines than normal cells. Until now, we have evidence of the production of only IL-3, GM-CSF and G-CSF from CML progenitor cells. The bystander myeloid cells were still observed to be massively expanded in IL-3 and GM-CSF-deficient mice with BCR/ABL-induced myeloproliferative disease (Ren 2002), suggesting that BCR/ABL may induce the production of other cytokines besides IL-3 and GM-CSF. However, the cytokine production induced by BCR/ABL in CML patients and in such a mouse model may be different, as it is not clear if the cell targets of transformation are the same.

The finding about IL-3 production lead to investigation of the possibility that BCR/ABL might activate IL-3 signaling in a ligand independent fashion (Wilson-Rawls, *et al* 1997). By using CML mouse model, bone-marrow cells from mice with a homozygous deficiency of IL-3Rβ_C or both IL-3Rβ_C and IL-3Rβ were incubated with p210 retrovirus and then injected into sublethally irradiated wild-type mice (Wong, *et al* 2003). The results showed a rapidly fatal CML-like illness occurs in all mouse models. The mice with the p210-transduced, wild type BM cells had a median survival of 12 days, the p210-transduced IL-3Rβ_C null BM cells survived 14 days, the IL-3Rβ_C(+/+)/IL-3Rβ(+/+)-reconstituted mice had a median survival of only 10 days and finally the p210-IL-3Rβ_C(-/-)/IL-3Rβ(-/-)-reconstituted mice still developed CML but significantly increased survival of 12 days longer (Wong, *et al* 2003). These studies suggest that BCR/ABL associated with IL-3R or GM-CSFR may not be required for signal activation to induce the murine CML-like myeloproliferative disorder. However, an important role for IL-3 and GM-CSF in human chronic phase CML or in minimal residual disease (Donato, *et al* 2001, Dorsey, *et al* 2002, Holyoake, *et al* 2001) including the promotion of survival of dormant marrow CML progenitors after imatinib therapy cannot be ruled out by these studies.

1.7.2. *Imatinib therapy of CML is not curative.*

The 2-phenylaminopyrimidines were first reported as potent protein tyrosine kinase inhibitors with selectivity for the ABL and the PDGF-R tyrosine kinases (Buchdunger, *et*

al 1996, Buchdunger, *et al* 1995). Imatinib emerged from these attempts as a lead compound for preclinical development. In the preclinical experiments, imatinib was shown to suppress the proliferation of BCR/ABL-expressing cells *in vitro* and *in vivo* (Druker, *et al* 1996). In colony-forming assays of peripheral blood or bone marrow from patients with CML, imatinib caused a 92-98% decrease in the number of BCR/ABL colonies formed, with minimal inhibition of normal colony formation (Druker, *et al* 1996).

Early clinical trials of imatinib were very promising, with selective target of killing of CML cells and little effect on normal progenitor cells. However, the ability of imatinib to cure chronic myeloid leukemia is questionable. There is evidence that CML patients have a leukemic population of non-cycling G_0 "quiescent stem cells" (Holyoake, *et al* 1999). The CML $CD34^+$ cells were able to exit G_0 and enter a cycling state, with subsequent up-regulation of IL-3 expression (Holyoake, *et al* 2001). Later it was confirmed that imatinib suppressed the growth of these malignant haemopietic progenitor cells by inhibiting abnormally increased cell proliferation, but did not affect the apoptotic rate of these cells (Holtz, *et al* 2002). A study on 15 patients with CML in chronic phase who were treated with imatinib to reach a complete cytogenetic response revealed that all patients still had evidence of BCR/ABL in their bone marrow $CD34^+$ cells detected by fluorescence *in situ* hybridization (Bhatia, *et al* 2003). The level of expression of BCR/ABL in these patients was higher in the $CD34^+$ cells than in the bone marrow mononuclear cells by quantitative PCR (Q-PCR). In addition, serial evaluation of these patients, despite continued treatment with imatinib, found persistence of BCR/ABL positive progenitors (Bhatia, *et al* 2003). Finally, a recent study by Graham *et al* showed that primitive, quiescent Ph-positive progenitor cells from CML patients are insensitive to imatinib *in vitro* (Graham, *et al* 2002).

1.7.3. *GM-CSF may maintain cell survival of CML progenitors and help CML progenitors to escape eradication by imatinib*

BCR/ABL containing cells delay apoptosis because of the constitutive tyrosine kinase activity which also leads to the stimulation of signals downstream of BCR/ABL, which are similar signals as those downstream of the IL-3 receptor. In addition, the defect of cell adhesion caused by the effect of BCR/ABL phosphorylation prevented mature

granulocytes from migrating out of blood circulation into inflammatory sites and also causes premature migration of granulocytes from bone marrow. Normal granulocytes in blood circulation have a life span shorter than the life span of extravasated neutrophils. Twenty-four hours after the onset of an inflammatory reaction, large numbers of neutrophils are still present at the inflamed site long after the cessation of neutrophil influx, typically noted at 1-4 hours (Bicknell, *et al* 1994). Intravascular cell number is regulated by the migration of granulocytes from bone marrow and the function of spleen to clear apoptotic cells when the cells pass through the spleen in the circulation. In CML, the circulation, migration and the clear-up function of spleen is unbalanced. One reason is that apoptosis of CML cells is delayed. BCR/ABL phosphorylation can maintain cell survival through PI3K/Akt pathways, but the decision of CML progenitor cells to enter maturation and apoptosis is not different from normal progenitor cells as shown in previous *in vitro* studies (Amos, *et al* 1995). These lead to the suggestion of a role for other factors which positively control cell apoptosis, such as cytokines.

A previous study showed that the activation of endothelium is important in recruiting neutrophils to sites of inflammation and in modulating their function (Coxon, *et al* 1999). Basically, the normal circulating neutrophils have a half-life of only 6-10 h and then execute a constitutive program of cell death (Bicknell, *et al* 1994). The onset of neutrophil apoptosis results in the loss of important neutrophil functions such as adhesion and phagocytosis (Dransfield, *et al* 1995, Whyte, *et al* 1993). However, the apoptosis of neutrophils could be delayed *in vitro* by the effect of inflammatory mediators such as GM-CSF, IL-6, and bacterial endotoxin(Brach, *et al* 1992, Colotta, *et al* 1992, Rossi, *et al* 1995). This study above showed that GM-CSF released by the activated endothelial cells is responsible for the delay of apoptosis of granulocytes. Again, studies in GM-CSF-defficient mice demonstrated that GM-CSF caused the delay in apoptosis of peripheral blood neutrophils but was not essential for the further delay in apoptosis of extravasated neutrophils, despite the presence of GM-CSF in the extravasation compartment(Coxon, *et al* 1999).

The evidence of the delay of apoptosis of normal neutrophils as above and the recent findings that cells containing detectable levels of BCR/ABL transcripts exist in the blood of a high proportion of normal adults(Biernaux, *et al* 1995, Bose, *et al* 1998) indicated that

the formation of BCR/ABL gene only might not be sufficient to produce disease and GM-CSF might play role in enhancement of the function of BCR/ABL. A number of further studies support this statement. Firstly, the study of Gunsilius *et al* (Takahashi, *et al* 1998) reporting that the BCR/ABL gene could be found in endothelial cells produced from a CML patient's cells. Secondly, although GM-CSF was controversially found to be produced by CML cells(el-Ahmady, *et al* 1997, Jiang, *et al* 1999, Jonuleit, *et al* 1998), its level was higher in both CML patients'serum and the serum of BCR/ABL-cell transplanted mice than IL-3 and G-CSF. Thirdly, IL-3, G-CSF and GM-CSF production has been found to associate with the level of BCR/ABL transcripts (Holyoake, *et al* 2001). It is possible that the high level of GM-CSF in patients' serum might come from the constitutive production of GM-CSF by endothelial cells. Taken together these suggested the possible role of GM-CSF to enhance the delay of apoptosis of CML $CD34^+$ cells.

1.8. Hypothesis and aim of the thesis

The signal transduction pathways common to BCR/ABL could be stimulated through cytokine receptors. These signals were hypothesized not to be inhibited by imatinib because of the specificity of imatinib itself for ABL kinase. If a cytokine GM-CSF, of which the receptor was hypothesized to stimulate all signals through BCR/ABL kinase, was applied in the cultures of CML cells, it should be able to stimulate such signals and support cell proliferation and survival even in cultures containing imatinib and also could enhance the spontaneous proliferative effect of BCR/ABL kinase.

Therefore, the general aim of the studies were to answer the questions,

1. Does GM-CSF modulate the effect of imatinib in terms of the proliferation and viability of CML $CD34^+$ cells?

2. Can CML cells produce GM-CSF which act in an autocrine fashion to protect cells from the action of imatinib?

3. Does GM-CSF signalling alter apoptosis pathways and modulate the ability of imatinib to induce cell death?

1.9. Summary

BCR/ABL protein can activate downstream targets of the IL-3 cytokine receptor superfamily group, including JAK2/STAT5, PI3K, and RAS pathways (Daley and Baltimore 1988, Kabarowski, *et al* 1994). GM-CSF is a member of the IL-3 receptor superfamily group, but its potential role in CML has been paid little attention, especially in terms of its functional effect as a single cytokine, and it has been reported to be produced naturally by other cells in the bone marrow such as fibroblasts and endothelial cells (Crosier, *et al* 1991, Gough, *et al* 1984, Wong, *et al* 1985). This cytokine is important in the regulation of the growth and development of normal hematopoietic cell population. GM-CSF, as a single cytokine, has been demonstrated to reduce the effect of imatinib (formerly called STI571 or CGP57148B) in terms of anti-proliferation, detected by a thymidine (TdR) uptake assay, in CML CD34 cells of some patients (Gambacorti-Passerini, *et al* 1997), although there was no statistical evidence to support this finding.

As GM-CSF and BCR/ABL share a number of downstream signalling pathways affecting cell proliferation and survival, we hypothesised that GM-CSF may be able to replace signalling of BCR/ABL when its tyrosine kinase activity is blocked by imatinib. It is clear that monotherapy with tyrosine kinases such as imatinib are unlikely to affect cure in CML, and cytokine signalling may be a rational point for intervention as an adjunct therapy.

Table 1.1. Inhibition of protein kinases by imatinib

Enzyme	*Substrate phosphorylation IC_{50} (μM)*	*Celllular tyrosine phosphorylation IC_{50} (μM)*
c-Abl	0.2;0.025*	
v-Abl	0.038	0.1-0.3
p210Bcr-Abl	0.025*	0.25
p185Bcr-Abl	0.025*	0.25
TEL-Abl		0.35
PDGF-R a and b		0.1
TEL-PDGF-R		0.15
c-Kit		0.1
FLt-3		>10
c-Fms and v-Fms	1.42 (Dewar et al, 2005)	
c-Src	>100	
v-Src	9	>10
c-Lyn	>100	
c-Fgr	>100	
Lck		
Syk (TPK-IIB)	>100	
Jak-2	>100	>100
EGF-R	>100	>100
Insulin receptor		>100
IGF-IR		>100
FGF-R1	31.2	
VEGF-R2	10.7	
VEGF-R1 (Flt-1)	19.5	
Tie-2 (Tek)	>50	
c-Met	>100	
PKA	>500	
PPK	>500	
PKCa, b, b2, g, d, z or h		
Protein kinase CK-1, CK-2	>100	
Cdc-2/cyclin B	>100	

IC_{50} was determined in immunocomplex assays.

IC_{50} means the concentration of imatinib causing a 50% reduction of kinase activity.

(Adapted from BJ Druker, 2002)

Table 1.2. Cells Expressing GM-CSF

Cell Type	Stimuli
Potential Physiologic sources of GM-CSF	
T Lymphocytes	antigen, lectin, CD28, IL-1, HTLV
B Lymphocytes	LPS, TPA
Macrophages	LPS, FCS, Phagocytosis, adherence
Mast cells	IgE, calcium ionophore
Fibroblasts	TNF, IL-1, TPA, modified LDL
Mesothelial cells	EGF + TNF
Osteoblasts	PTH, LPS
Potential Pathophysiologic Sources of GM-CSF	
AML	TNF, adherence, IL-1
Rheumatoid synovium	
Soloid tumors	

Abbreviation: LPS, lipopolysaccharide; FCS, fetal calf serum; EGF, epidermal growth factor; LDL, low-density lipoprotein; PTH, parathyroid hormone.

(Adapted from Judith C Gasson, 1991)

Chapter 2.

Materials and methods

2.1. Suppliers of commonly used reagents

Table 2.1. Commercial reagents and their suppliers

Reagent	Supplier	Catalogue Number
2-Ethoxyethanol	APS Fine Chemicals	211-500M
7-aminoactinomycin D (7-AAD)	Molecular Probes	A-1310
14-3-3	SANTA CRUZ	F2904
α-Naphthyl butyrate	Sigma®	N-8000
β-Mercaptoethanol	Sigma®	M-6250
Acrylamide (40% bis solution)	Bio-Rad	161-0148
Akt1 (B-1)	SANTA CRUZ	sc-5298
Albumin bovine serum, Cohn Analog, min. 98%	Sigma®	A1470-100G
Apotinin	Roche	0236624
Bacto™ Agar	Difco	214010
Bad	SANTA CRUZ	sc-942
Bcl-XL (H5)	SANTA CRUZ	sc-8392
BIM (H-191)	SANTA CRUZ	sc-11425
Micro BCA™ Protein Assay kit	PIERCE	23235
Bovine Serum Albumin (BSA) powder (Fraction V)	Sigma®	A9418
CD34 (8G12) PE	BD Bioscience	348057
Disodium hydrogen phosphate (Na_2Hpo_4)	Aldrich	219886-500g
Dimethyl sulphoxide	APS Fine Chemicals	747-500mL
E21R	BresaGen Limited	461700
Erk (1/2)	SANTA CRUZ	H0504

Reagent	Supplier	Catalogue Number
Ethylene Diamine Tetra-Acetic Acid Di-Sodium Salt (EDTA)	APS Fine Chemicals	180-500g
Ethylenedioxybis Tetra-Acetic Acid	SANTA CRUZ	sc-3539
Ethanol (analytical grade)	AnalaR®, BDH	10107.2500P
Fast Blue BB Salt	Sigma®	F-3378
Fast Garnet GBC	Sigma®	F-8716
Ficoll-Hypaque (Lymphoprep™ 1.077 g/dL)	Axis-shield Poc, Oslo, Norway	1114547
Foetal bovine serum (FCS)	JRH Bioscience	12103-500M
Glucose	Sigma®	G-7528
Glutaraldehyde	Sigma®	G-6403
Glycerol (analytical grade)	Ajax Finechem	A242-500ML
Hanks balanced salt solution (HBSS)	JRH Biosciences	55021-500M
HEPES (1M)	JRH Biosciences	59205-100M
Holo-transferrin	Sigma®	T-0665
Hybond-P PVDF Membrane	Amersham Biosciences	RNP303F
Insulin (Actrapid® 100 IU/mL)	Novo Nordisk Pharmaceuticals	Actrapid® 10mL
Iscov's Modified Dulbecco's Medium (IMDM)	JRH Biosciences	51471-500M
Leupeptin	Roche	1017101
L-glutamine	JRH Biosciences	59202-100M
Lauryl sulphate (SDS)	Sigma®	L-4509
Lipoprotein (Low Density)	Sigma®	L-7914

Reagent	Supplier	Catalogue Number
Luxol Fast Blue MBS	Gurr®, BDH	34044 3P
Methyl Violet	AnalaR®, BDH	34033
Mouse IgG_1/RPE (control)	DAKO	X0298
N,N-Dimethyl formamide	APS Fine Chemicals	2217-500M
N,N,N',N'-Tetramethylethylenediamine (TEMED) Approx. 99%	Sigma®	T-7024
Naphthol AS-D Chloroacetate	Sigma®	N-0758
Nonidet® P40 Subtitute (NP-40)	Fluka BioChemika	74385
p-Akt (ser 473)	SANTA CRUZ	F 0905
Paxillin antibody	Cell signaling technology®	2542
Penicillin G (5000 U/mL)-Streptomycin sulphate (5000 µg/mL)	CSL Biosciences	05081901
p-Erk(1/2) (E-4)	SANTA CRUZ	H1104
Phorbol 12-myristate 13-acetate (PMA)	Sigma®	P1585
Phospho Plus® Bad (Ser112/136) antibody kit	Cell Signaling technology®	9290
Sodium Cholride	APS Fine Chemicals	465-2.5kg
Sodium fluoride, SigmaUltra, minimum 99.0 %	Sigma®	S7920-100G
Sodium orthovanadate (minimum 90% titration)	Sigma®	S6508-50-G
Sodium pyrophosphate tetra-basic decahydrate, SigmaUltra, minimum 99.0 %	Sigma®	S6422-100G
Tris (Sigma-7-9®)	Sigma®	T-1378

Reagent	Supplier	Catalogue Number
Trypan blue (0.4%)	Sigma®	T-8154
Tween-20	Sigma®	P-9416
Urea	APS Fine Chemicals	817-5kg

2.2. Buffers and solutions used in cell culture

The composition of all buffers and solutions used throughout this thesis is described below. All solutions were made using sterile dH_2O or RPMI 1640 or HBSS, and stored in a 4°C fridge unless otherwise indicated.

2.2.1. *0.66% Bacto™ agar*

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

0.33 gm of Bacto™ agar powder was subjected to 50 ml of sterile Milli-Q® water (Millipore Corporation, USA) without stirring in a 100mL bottle. Then this mixture was autoclaved at 121 °C for 20 minutes on a fluid cycle. After cooling the autoclave down to about 80-100°C with the autoclave pressure reduced to zero paskcal (approximately 30 minutes), the mixture was taken out of the autoclave and swirled to ensure mixing. Then it was kept at room temperature until use.

For colony assays, the agar was melted by heating in a microwave for 45 seconds, swirling thoroughly, and then re-heating for further 15 seconds. After that, the agar was retained in the waterbath at 41 °C all the time until used.

2.2.2. *Agar-Iscov's Modified Dulbecco's Medium (A-IMDM)*

FCS	25.0	mL
2x IMDM	25.0	mL

L-glutamine	500.0	µL

After mixing all constituents, the solution was sterilized with a 0.2 µm single use syringe filter (Satorius Minisart) just before use. Then the solution was retained at 37 °C in the waterbath until use.

2.2.3. *Blocking Buffer (HBSS/0.8% BSA/ 4% NHS/ 5% FCS)*

HBSS	500.0	mL
BSA powder	4.0	g
Normal human serum	20.0	mL
FCS	25.0	mL

The BSA was added to the HBSS without stirring to allow it to be dissolved slowly at room temperature. Then the solution was added with normal human serum and FCS. To sterilizing the final solution, the solution was passed through a 0.2 µm bottle top filter. Then the blocking buffer was kept at 4°C.

2.2.4. *CFSE preparation*

CFSE stock was prepared by dissolved CFSE powder in sterile technique with DMSO to the concentration of 5 mM. Then 50 µL of CFSE stock was aliquoted to a 1.5-ml eppendorf tubes and kept in -20°C freezer. The CFSE working solution was prepared by thawing an eppendorf of CFSE at a time and then adding 450 µL of cold PBS to CSFE. This makes the final concentration of CFSE to 0.5 mM. To label cells with CFSE, 20 µL of CFSE working solution was added to 1 ml of cells containing cell number up to $5x10^7$ cells, giving a final CFSE concentration of 10 µM).

2.2.5. *CFSE quenching buffer*

PBS	500.0	mL
FCS	25.0	mL

An aliquot of frozen FCS was thawed and subjected to 500 mL of sterile PBS (IDL store, IMVS). The solution was sterilized using a 0.2 μm bottle top filter and stored at 4 °C.

2.2.6. *Double-Strength Iscov's Modified Dulbecco's Medium (2x IMDM)*

IMDM powder	1.0	portion (17.67 g)
L-glutamine	0.4	g
L-asparagine	0.2	g
$NaHCO_3$	6.048	g
Milli-Q® water		

IMDM powder was dissolved in 400 mL Milli-Q® water, and then supplemented with L-glutamine, L-asparagine and $NaHCO_3$. All the ingredients were dissolved thoroughly with the aid of a magnetic stirrer. Then more Milli-Q® water was added to the solution to adjust volume to 500 ml. The final solution was sterilized with a 0.2 μm bottle top filter and aliquoted to 25 mL stored at -20 °C.

2.2.7. *1.5% glutaraldehyde Fixation*

50% Glutaraldehyde Stock	3.0	mL
Milli-Q® water	97.0	mL

3 ml of 50% glutaraldehyde solution was added to 100 ml of dH_2O. The mixture was mixed well before being stored at room temperature. The glutaraldehyde fixation suspension was prepared fresh for each experiment.

2.2.8. *HBSS/HEPES/FCS*

Hanks Balanced Salt Solution	500.0	mL
HEPES (1M)	5.0	mL
FCS	25.0	mL

This medium is used to supplement and wash cell suspension after ficolling. Sterile HEPES and FCS were added to HBSS and then stored at 4 °C.

2.2.9. *Imatinib (10 mM stock)*

Imatinib mesylate was a kind gift of Novartis, Basel, Switzerland. In some cases, imatinib mesylate was purified from commercially obtained Glivec capsules.

Imatinib powder	0.051	g
Milli-Q® water	8.648	mL

Imatinib powder (Novartis, Basel, Switzerland) was dissolved in Milli-Q® water and then sterilized with a 0.2 μm single use syringe filter. The solution was stored at -20 °C until use. The titration range between 0 to 10 μM imatinib was used to examine cell proliferation in the presence of cytokine GM-CSF and its receptor antagonist E21R to compare the effect of GM-CSF on cells among these dosage points. The doses of 400 mg and 600 mg imatinib daily, which has been reported sufficient to suppressed the CML, were equivalent to an *in vitro* concentration of approximately 1.0-1.9 μM and 3.7 μM respectively (Bakhtiar, *et al* 2002, le Coutre, *et al* 2004)

For the convenient use, 1 part of 10 mM imatinib stock was subjected to 9 parts of Milli-Q® water to make 1mM or 1000 μM imatinib stock. To make 100 and 10 μM stock, 1 part of 1000 and 100 μM was subjected to 9 parts of the Milli-Q® water respectively. To perform cell cultures, the added imatinib volume should not be greater than 5% of total volume. Otherwise, the designed concentration of imatinib might be underestimated. The stock and volume of imatinib used in cell culture were shown in table 2.2.

Table 2.2. The appropriate volume of imatinib used in cell cultures

Doses of Imatinib (μM)	For cell volume of 100 μL		For cell volume of 200 μL		For cell volume of 1 mL		For cell volume of 5 mL		For cell volume of 10 mL	
	Imatinib volume (μL)	Stock (μM)	Imatinib volume (μL)	Stock (μM)	Imatinib volume (μL)	Stock (μM)	Imatinib volume (μL)	Stock (μM)	Imatinib volume (μL)	Stock (μM)
0.1	1	10	2	10	10	10	5	100	10	100
0.3	3	10	6	10	30	10	15	100	30	100
1	1	100	2	100	10	100	5	1000	10	1000
3	3	100	6	100	30	100	15	1000	30	1000
10	1	1000	2	1000	10	1000	5	10mM	10	10mM

2.2.10. *IMDM/1%BSA*

IMDM	500.0	mL
BSA powder	5.0	g

The BSA was dissolved slowly at room temperature in IMDM without stirring. The solution was sterilized with a 0.2 μm bottle top filter and stored at 4 °C.

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

HBSS	449.0	mL

BSA	2.5	g
EDTA (1M stock)	1.0	mL

BSA was dissolved slowly at room temperature without stirring in HBSS. Then EDTA was added to the buffer. The final solution was filtered to sterilize with 0.2 μm bottle top filter and kept at 4 °C.

2.2.12. *Medium for culturing K562, KU812, MOLM-1 and Meg-01*

RPMI 1640	500.0	mL
FCS	50.0	mL
L-Glu (200mM)	5.0	mL
Penicillin (5000 U/ml)/ Streptomycin sulphate (5000 μg/mL)	5.0	mL

All constituents were thawed in a 37 °C-waterbath before pouring in 500 mL RPMI. The final solution was sterilized with a 0.2 μm bottle-top filter and stored at 4 °C until use. The solution should be warmed in a 37°C-waterbath for 10 minutes when performing cell cultures.

2.2.13. *Medium for culturing CTLL (WT for hGM-CSFR α and β) and CTL-EN (positive for hBCR/ABL and hGM-CSFR α and β)*

RPMI 1640	500.0	mL
FCS	50.0	mL
L-Glu (200mM)	5.0	mL
Penicillin (5000 U/ml)/ Streptomycin sulphate (5000 μg/mL)	5.0	mL
Mouse IL-2	55.0	μL
β-mercaptoethanol (0.1M)	200.0	μL

All constituents were thawed in a 37 °C-waterbath before pouring in 500 mL RPMI. The final solution was sterilized with a 0.2 μm bottle-top filter and stored at 4 °C until use. The solution should be warmed in a 37°C-waterbath for 10 minutes when performing cell cultures.

2.2.14. *2-mercaptoethanol (1M)*

β-mercaptoethanol (14.27M)	700.0	µL
PBS	9.3	mL

β-mercaptoethanol was diluted in PBS (IDL unit, IMVS) and stored at -20 °C. This stock was further diluted 1:10 in PBS to give a concentration of 0.1 M as a working solution.

2.2.15. *Normal human serum*

Normal human serum was from the donated peripheral blood which was heparinized. The blood was left standing at room temperature until the clear serum was layered separately. The clear serum was decanted to a new sterile 50-ml conical tube and was heated inactivated at 56 °C for 45 minutes. Ten millilitre (10 mL) aliquots were stored at -20 °C.

2.2.16. *0.067 M Phosphate Buffer, pH 6.3(1L)*

KH_2PO_4	7.0	g
Na_2HPO_4	2.2	g
Milli-Q® water		

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

2.2.17. *0.067 M Phosphate Buffer, pH 7.4 (1L)*

KH_2PO_4	1.74	g
Na_2HPO_4	7.68	g
Milli-Q® water		

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

2.2.18. *Serum Deprived Medium (SDM (10 mL)*

IMDM/1% BSA	10.0	mL
L-glutamine (200 mM)	100.0	μL
Insulin (1 mg/mL)	100.0	μL
Transferrin (20 mg/mL)	100.0	μL
Lipoprotein (low density)(12.5 mg/mL)	40.0	μL
2-mercaptoethanol (0.1 M)	5.0	μL

The solution was sterilized with a 0.2 μm single use syringe filter. The combination of these constituents resulted in the final concentration to be 2 mM L-glutamine, 200 μg/mL transferring, 10 μg/mL insulin, 10^{-4} M β-mercaptoethanol, and 50 μg/mL low density lipoproteins. The solution was stored at -20 °C.

2.2.19. *Thaw Solution*

IMDM	400	mL
FCS	100	mL
L-asparagine	0.1	g
L-glutamine	5.0	mL
DNAase	25000	units

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

2.2.20. *Urea Saturated Ethanol*

Urea was added to 70% ethanol at room-temperature until a saturated solution was attained. The solution was stored indefinitely at room temperature.

2.2.21. *While Cell Fluid*

Acetic acid	2.0	mL
Milli-Q® Water	98	mL
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 0.2 μm 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*

2.3.1.1. *Cryopreservation of cell lines*

The cell lines will be cultured in the medium as described above. After reaching the exponential growth, total cell number was about $5x10^7$ to $1x10^8$ cells in total. Cells were washed twice with 10% FCS/RPMI, and then were re-suspended with fresh RPMI only at the density of $1x10^7$ cells / mL. Two times cryopreservation reagent, containing 20% DMSO, 40%FCS and 40% RPMI were fresh prepared and kept on ice before used. Then the equal volume of cryopreservation reagent was drop-wise added to the cell suspension with constant mixing. The final cell suspension was transferred quickly to cryo-ampoules (Nalgene®) and the samples stored in liquid nitrogen (-196 °C). The final concentration of cells was $5x10^6$ cells/mL.

2.3.1.2. *Thawing cell lines*

K562, KU812, MOLM-1, Meg-o1, CTLEN and wt-BCR-ABL / CTLEN cells kept frozen in liquid nitrogen were thawed rapidly by immersion in a 37°C waterbath with manual agitation followed by the transfer of the suspended cells into a 50-ml Falcon tube by pasteur pipette. An equal volume (1-2ml) of the prewarmed RPMI 1640 supplemented with 10% FCS was added drop-wise. After left standing for 2 minutes, another equal volume of above medium was added drop-wise to the cell suspension followed by a further

2-minute stand. Then the Falcon tube was slowly filled with the remaining pre-warmed-medium as above to a final volume of 50 ml.

After pelleting cells by centrifugation for 5 minutes at 1400 rpm at room temperature, the supernatant was discarded and the cells were washed twice in 20-25 ml of the prewarmed medium above. After last wash all cell lines were suspended with 5 ml of RPMI 1640 supplemented with 10% (v/v) heat activated, sterile FCS together with penicillin G 5000 U/ml/ streptomycin sulfate 5000 µg/ml and 2 mM L-Glutamine. Cell density and viability were determined by trypan blue exclusion. Cells then were cultured in small (25 cm^2) tissue culture flask and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were subcultured into 75cm^2 polycarbonate tissue culture flasks when cell viability neared 100% (around 2 days) at a density of $5x10^5$ cells/mL. All cell culture manipulation was performed in an UV-Oliphant Laminar Flow Hood. Cells in cultures were maintained and adjusted to the density of $2x10^5$ cells/mL after the first subculture above.

2.3.1.3. *Performing the cell cultures*

Non-adherent cell lines containing K562, MOLM-1, KU812, Meg-o1, CTLEN with complete human GM-CSF receptors, and wt-BCR-ABL/ CTLEN with complete human GM-CSF receptors and BCR/ABL were maintained at a cell density between 5.0 x 10^4 to 1.0 x 10^6 cells /mL in 25cm^2, 75cm^2, or 150cm^2 tissue culture flask (Greiner). Medium was pre-warmed to 37 °C prior to use.

For CTLEN with human complete GM-CSF receptors, cells was cultures in medium RPMI containing m-IL-2 at diluition 1: 10,000 of original stock supplemented with 10% FCS, 2 mM L-Glu, Penicillin G (50 U/mL)/ Syreptomycin sulphate (50 µg/mL), and 0.1 M β-Mercaptoethanol (1: 2000).

Other cell lines were cultured in RPMI supplemented with 10% FCS, 2 mM L-Glu, and Penicillin G (50 U/mL)/ Streptomycin sulphate (50 µg/mL).

For starving cell overnight (about 12-16 hours) before performing cell cultures to get cell lysate for Western Blot, all types of cells were cultures in RPMI supplemented with 0.5% FCS, 2 mM L-Glu, and Penicillin G (50 U/mL)/ Streptomycin sulphate (50 µg/mL). Then the same starving medium was used to culture cells under indicated conditions.

2.3.2. *Maintenance of primary CML cells*

2.3.2.1. *Technique of ficolling primary CML cells*

Cells from 5 donors and 23 patients who were diagnosed to be CML in chronic phase as described in table 2.1 were ficolled by Lymphoprep to get mononuclear cells. Five ml of patient peripheral blood or bone marrow were diluted in 25 ml of HBSS containing 5 mM HEPES in a 50-ml Falcon tube. Fifteen ml of Lymphoprep was gently added to the bottom of the diluted blood by using suction canula. The mononuclear cells were washed twice with HBSS supplemented with 5 mM HEPES. The mixture was centrifuged at 400g (~1200 rpm) for 30 minutes. The layered mononuclear cells were carefully and gently suctioned with pasteur pipette and transferred to another sterile 50-ml falcon tube. Then the mononuclear cells were washed twice with 50 ml of HBSS/5mM HEPES. After last wash, mononuclear cells were suspended with 5 to 10 ml of the same media and cell number and viability was performed.

2.3.2.2. *Cryopreservation of the primary CML cells*

The mononuclear cells were added with the same media as above to the density of $3x10^7$cells/ml. An equal volume of freezing mix containing 40% (v/v) HBSS/5mM HEPES, 40% FCS (v/v) and 20% DMSO (v/v) was slowly dropwise added to the mononuclear cell suspension. This made the final cell density to $1.5x10^7$cells/ml and the final DMSO concentration to 10% (v/v). Cells will be put into ice and transfer to the rate freezer immediately after adding DMSO to the ficolled cells. This will take about an hour for the rate freezer to decrease the temperature down to -80°C. Then the frozen cells were transferred to keep in liquid nitrogen.

2.3.2.3. *Thawing the primary CML cells*

Cells were thawed rapidly at 37^{0}C, and transferred to a 50-ml centrifuge tube. Pre-warmed HBSS plus 5% FCS plus 5% ACD (anticoagulant-citrate-dextrose), was added slowly drop-wise until the total volume was 50 ml. The cells were centrifuged at 400g for 5 minutes, and re-suspended at a final volume of 5 ml, and cell count and viability were determined.

2.3.2.4. *Performing the cultures of primary CML cells*

The primary CML cells after thawing will be re-suspended and wash twice in 20-50 mL 0.1% BSA in PBS for the CFSE staining. After last wash, cells were resusupended in 0.1% BSA/PBS at a density up to 5x10^7 cells/mL. Then CFSE will be added to the cell suspension at the final concentration of 10 μM during gentle vortex. Then cells will be incubated in the waterbath at 37 °C for 10 minutes in the dark with 2 or 3 times of gentle shaking. After incubation, the labeled cells will be washed and the reaction of cells on CFSE will be quenched using 5 folds of cell volume of cold 10% FCS / PBS thrice. Then the cells will be re-suspended in SDM prepared as described above overnight in the humidified incubator at 37 °C, at quite high density of about 1x10^6 cells /mL. In the next day, cells will be washed thrice in cold 10% FCS/ PBS before being re-suspended with the same medium about 5x10^7 cells/mL. The labeled cells are then stained with 1 μg of 7-AAD and PE-conjugated CD34 for the cells number up to 1 x 10^6 cells / mL before performing sorting on FACStar to sort 7-AAD$^-$ / CD34$^+$ cells within the 40-channel fluorescence gate on CFSE.

The sorted cells will be seeded in the 96-well or 24 well-plates and cultured in SDM at the density of not greater than 5 x10^5 cells per mL.

2.4. *General cell washing*

For large volume of cell suspensions, cells were centrifuged in a Heraeus multifuge 3$_{S-R}$ benchtop centrifuge at 420g (~ 1400 rpm) for 5 minutes at room temperature. For ficolling original blood or bone marrow, after layering blood or bone-marrow sample with Lymphoprep, the mononuclear cells were separated via the Heraeus mutifuge as above at 400g (~ 1200 rpm) for 30 min without brake setting at room temperature.

For the small cell volume for example cell washing following fluorescent antibody staining, cells were washed at room temperature by centrifuging for 2 min. at 815 g (~2750 rpm) in a DiaCent-12 DiaMed mini centrifuge.

2.5. *General cell number and viability evaluation*

One milliliter of evenly mixed cell suspension was transferred to a 1.5-ml eppendorf. Ten to twenty micro-milliliter of this cell suspension was added with an equal volume of 0.8% (v/v) trypan blue solution (a 1:2 dilution). Then 10 μL of cells/tripan blue suspension was transferred to haemocytometer counting chambers (Neubauer Improved, Assistant, Germany). Then cell viability and number were assessed through the Olympus microscope at 10x magnification.

2.6. *Immunofluorescence stain and flow cytometric analysis*

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

2.6.1. *Staining for flow cytometric analysis*

A known number of cells were transferred to 5 mL round bottom polystyrene tubes (Falcon) and suspended in a minimum volume (~ 50 μL) of 5%FCS/PBS. Appropriate antibodies or staining such as 7-AAD were added to tubes at the concentration of 1μg / 10^6 cells unless otherwise stated , and incubated for a period of 45 minutes on ice in the dark. The fluorescence intensity of cell suspensions was examined using an Epics® -XL-MCL flow cytometer and WinMDI Version 32 software (Verity software). 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.

2.6.2. *Carboxyfluorescein Diacetate Succinimidyl Ester Labeling of Cells*

Carboxy fluorescein diacetate succinimidyl ester (CFDA-SE or CFSE) is a fluorescein derivative that is cell permanent and non-fluorescent. The cleavage of acetate groups by intracellular esterases renders the molecule fluorescent, and the attachment of fluorecein groups to proteins via aminolysis of the succimidyl ester ensures that the dys is retaimed within the cells. As cells divide, CFSE is distributed equally between daughter cells (Lyons and Parish 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 labeling of up to $5x10^7$ cells, 20 µL of 5mM CFSE stock (in DMSO) diluted to 1: 10 in PBS was used. This CFSE solution was added to cells that had been suspended in 1 mL ice-cold 0.5% BSA in PBS, giving a final concentration of 10 µM CFSE. The cell suspension was vortexed thoroughly and incubated at 37 °C in a waterbath for 10 minutes. Excess CFSE was quenched by adding 3 mL cold 10% FCS in PBS and centrifuging for 2 minutes at 815g in a DiaCent-12 mini centrifuge (DiaMed). Cells were washed thrice with cold 10% FCS in PBS prior to culture or sorting on flow cytometer.

Due to hetrogenous fluorescence profile, CFSE labeled $CD34^+$ 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(Nordon, *et al* 1997).

CFSE division analysis was performed using an Epics® -XL-MCL cytometer and Cytomics^{TM} 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 division cycle was determined using a marker tool which was set according to the maximum fluorescence of undivided, non-stimulated controls (using 100 ng/mL colcemid to inhibit cell division).

2.6.3. *Isolation of $CD34^+$ cells by MACS Separation of CD34 progenitor beads*

$CD34^+$ cells were separated from MNC from BM or peripheral blood of donors and CML patients after ficolling. 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 300 µL MACS $CD34^+$ buffer per 10^8 cells, and 100 µL 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-R} bench top centrifuge and re-suspended in 400 µL MACS buffer per 10^8 cells. MACS anti-hapten microbeads (100 µL 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 then were washed twice with MACS $CD34^+$ buffer using a Heraeus multifuge 3_{S-R} bench top centrifuge, and then re-suspended in 500 µL degassed MACS buffer for cells up to 10^8 cells.

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

The purity of $CD34^+$ cells following the isolation procedure was checked by staining with an anti-CD34-PE, as described in 2.6.1.

2.7. *Haemopoietic colony assays*

2.7.1. *Establishment of colony cultures*

CML $CD34^+$ cells were assayed their ability to form colonies on semi-solid gar, based on a modification of a method previously described by Johnson *et al.* (1980)(Johnson 1980). The modified method has been done on cell-fluid culture after 3 days of cultures under conditions containing imatinib, GM-CSF and the GM-CSFα-receptor specific blocking agent E21R. This aims to evaluate the left colony number after treatment. Therefore, $2x10^5$ cells/ mL of CML $CD34^+$ cells were seeded in SDM supplemented with imatinib over the range 0-10 μM ± 300 pg/mL GM-CSF ± 10 μg/mL E21R. Cells were cultured in 24-well plates for 3 days, and then were harvested to 5-mL poltpropylene tubes, washed twice with 0.1% BSA/IMDM, and then re-suspended with 1mL 1x IMDM with 50%FCS and and keep warm sterilely in 37 °C waterbath. Meanwhilst, 4 mL of cell-agar suspension was prepared by combining 2 mL of 0.66% agar (held at 41 °C), 1mL of 1x IMDM/50% FCS (held at 37 °C) and 1 mL of cell suspension which has already warmed in the 37 °C waterbath respectively. Then the cell-agar suspension was swirled quickly, and 1 mL aliquots dispended onto pre-prepared 35 mm cell culture dishes, ensuring that no air bibbles 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 and 5% CO_2 for aperiod of 14 days. The tripicate control of the same cells has been done on the non-fluid cultured cells at $2x10^5$ cells in 4 mL of cell-agar suspension and cultured onto the 35 mm cell cultures dishes for 14 days.

Pre-preparation of 35 mm cell culture dishes involved dispensing growth factors containing 10 ng/mL each of G-CSF, IL-3, TPO, Fl3-ligand, SCF and IL-6 (Prepotech) in order to promote maximal colony forming.

2.7.2. *Fixation of colony assay cultures*

After 124 days in culture, colony cultures were fixed by the addition of 1 mL of 1.5% glutaraldehyde, and left for 3 days at 4 °C. Fixed agar cultures were then rinsed with PBS and transferred to glas slides using water-floating technique. This was performed by floating the agar plugs in PBS and covering the top of culture dish with a glass slide (75mm x 50mm). The slide/dish was then carefully flipped 180° under PBS level in the PBS tray. The slide and disk will separated easily under PBS. 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.

2.7.3. *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 (≥ 50 cells per colony).

2.7.3.1. *Naphthol acetate esterase staining*

Twenty-five millilitres (25 mL) of 2-etoxyethanol was combined with 25 drops of α-naphthol butyrate in a 1 L beaker. Briefly, 1 L of 0.067 M phosphate buffer pH 6.3 and 0.5 g 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 room temperature (RT), and then washed thoroughly with tap water.

2.7.3.2 *Chloroacetate esterase staining*

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

2.7.3.3. *Luxol fast blue dye*

Luxol fast blue at 1.5 g was dissolved in 1 L of saturated Uread in 70% ethanol. The solution was filtered using Whatman paper, and slides stained for a period of 2 hours at

RT. Slides were thoroughly with tap water and then mounte in DePex mounting medium (Gurr® BDH).

2.7.3.4. *Absolute number evaluation by Flow Beads*

Flow-Check™ fluorosheres (Beckman Coulter) were washed twice inHBSS/0.1% BSA and re-suspended at $1x10^6$ beads/ mL. Five thousand or 5µL flow beads were added to each well of cell fluid culture before harvesting. Then the cultures were well mixed and transferred to the 5 mL polypropylene tube to perform other staining. Before performing flow-cytometry, 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 vs 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:

$$\left[\frac{\text{No. events in bead gate}}{\text{No. events in cell gate}}\right] = \left[\frac{\text{bead number}}{\text{Cell number}}\right]$$

2.8. ***Protein analysis***

2.8.1 *Molecular Biology reagent*

2.8.1.1.*Loading buffer, 4X Denaturing*

Tris-HCl (0.5M, pH 6.8)	250	mM
SDS (40%)	8%	
Glycerol (100%)	40%	
β-mercaptorthanol	20%	
Bromophenol Blue (1%)	0.075%	

1 mL aliquots of this combination were stored at – 20 °C.

2.8.1.2. *Lysis Buffer*

1% NP-40 in TSE	7.6	mL
NaF (0.5M)	200	µL
Na-pyrophosphate(0.1M)	1.0	mL
Sodium vanadate (0.5M)	200	µL
Complete protease inhibitors (optional)	400	µL
Leupeptin (1mg/mL)	200	µL
Apotinin (1mg/mL)	200	µL
PMSF (0.1M)	200	µL

Lysis buffer was freshly prepared only before use and kept on ice or kept at 4 °C.

Complete protease inhibitors (Roche Diagnostics), was prepared by adding 1 cocktail tablet to be dissolved in 1 mL Milli-Q® water, the final solution = 50 times concentration. The solution was stored at – 20 °C.

PMSF was added just prior to lysis.

2.8.1.3. *Membrane blocking solution*

Bovine serum albumin	5	g
0.1% Tween 20/1x TBS	100	mL
EDTA	1	mM

The solution was prepared fresh on the day of use.

2.8.1.4. *5% Polyacrylamide Gel (Stacking Gel)*

Milli-Q® water	3	mL
Acryl (40%)	630	µL
Tris (0.5M)	1.26	mL
SDS (10%)	50	µL
APS (10%)	50	µL
Temed	5	µL

All reagents were combined just before pouring of the gel.

2.8.1.5. *10% Polyacrylamide Gel (Resolving Gel)*

Milli-Q® water	4.3	mL
Acryl (40%)	2.5	mL
Tris (0.5M)	2.5	mL
SDS (10%)	100	µL
APS (10%)	100	µL
Temed	6	µL

Reagents were prepared just before pouring of the gel.

2.8.1.6. *SDS-PAGE Electrode Buffer (25 mM Tris/ 192 mM Glycine/ 0.1% SDS)*

Tris	3.026	g
Glycine	14.413	g
SDS	1.0	g
Milli-Q® water	make volume up to 1 L	

Tris, glycine and SDS were dissolve in Milli-Q® water with the aid of a magnetic stirrer. If all the reagents are accurately weighed, the Ph should be 8.3.

2.8.1.7. *TBS stock (10X)*

Tris	24.2	g
NaCl	87.6	g
Milli-Q® water	make volume up to 1 L	

The reagents were combined in approximately 800 mL Milli-Q® water with the aid of magnetic stirrer. Then pH was adjusted to 7.5 before making the volume up to 1 L with Milli-Q® water.

2.8.1.8. *IX TBS*

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

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

2.8.1.9. *TBS-T (1x TBS / 0.1% Tween)*

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

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

2.8.1.10. *TE Buffer (10x)*

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

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

2.8.1.11. *Transfer Buffer(25mM Tris, 192 mM glycine, 30% methanol)*

Glycine	14.413	g
Tris	3.026	g
Methanol	300	mL
Milli-Q® water	make up to 1 L	

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

2.8.1.12. *1.5M Tris (pH 8.8)*

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

Tris was dissolved in approximately 80 mL Milli-Q® water and then adjusted pH to 8.8 with concentrated HCl before making the volume up to 100 mL. The solution was stored at room temperature.

2.8.1.13. *0.5M Tris (pH 6.8)*

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

Tris was dissolved in approximately 80 mL Milli-Q® water and then adjusted pH to 6.8 with concentrated HCl before making the volume up to 100 mL. The solution was stored at room temperature.

2.8.1.14. *TSE Buffer (50mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0)*

Tris	6.052	g
NaCl	8.760	g
EDTA	0.372	g

Tris, NaCl and EDTA was dissolved in approximately 950 mL of Milli-Q® water, and then adjusted pH to 8.0 before making the volume up to 1 L with Milli-Q® water.

2.8.2. *Method of Western Immunoblot*

2.8.2.1. *Western Immunoblot with the total protein lysate (all the process has been performed on ice or at 4 °C)*

Cells were washed thrice with the starved medium then were cultured with starved medium at the density of $5x10^5$ cells / mL overnight for the cell line, and only 4 hours for the thawed primary CML cells to let cell recover in the 37 °C humidified-incubator with 5% CO_2. Cells at the density $5x10^5$ to $1x10^6$ cells / mL were incubated for 2 hours in starved medium or otherwise as indicated with imatinib only, then were stimulated with GM-CSF for 10 minutes just before harvesting. Cell reaction was stopped by washing with 50 mL cold PBS thrice. Then the cell pellet will be lysed with the 1% NP-40 lysis buffer at the cell density of $2x10^5$ cells for the cell line or $2x10^6$ cells for the primary CML cells in

15μL of lysis buffer. The cell suspension was incubated on ice for 30 minutes then centrifuged at 16,000xg for 20 minutes at 4 °C to get rid of all cell fragment. After that, 5 μL of 4x loading buffer was added to the cell lysate. The final lysate was well mixed and boiled for 5 minutes before loading in the SDS-PAGE gel.

2.8.2.2. *Western Immunoblot of the immunoprecipitate protein (all the process has been performed on ice or at 4 °C)*

Cell cultures were performed as described in 2.8.2.1. Then cell reaction was stopped by washing cell with 50 mL cold PBS. Then cell lysate was performed using 1 mL of 1% NP-40 / TSE for $1x10^7$ cells of cell line or $1x10^8$ cells of primary CML cells and incubated on ice for 30 minutes. The lysate was then centrifuged at 16,000xg for 20 minutes at 4 °C to remove all cell fragments . Then 500 μg of protein or equal amount of protein from cell lysate measured by BCA assay was transferred to the new eppendorf. Then 1μg/mL of the specific antibody was added to the lysate and incubated overnight at 4 °C. In the next day, 50 μL of Protein A Sepharose™ 4 Fast Flow (Amersham Biosciences) per 1 mL of cell lysate was added to the cell lysate and the suspension was incubated at 4 °C for 4 hours. Then the lysate/beads suspension was centrifuged at 10,000xg for 5 minutes before being re-suspended with 30 μL of 1% NP-40/TSE. Then 10 μL of 4x loading buffer was added to the lysate, and the mixture was boiled for 5 minutes before loading to the SDS-PAGE gel.

2.8.2.3. *Preparation of subcellular fractions*

Subcellular fractionation was performed as described in Salomoni *et al.* 1998(Salomoni, *et al* 1998). Firstly, cells were lysed in the hypotonic buffer, containing 5 mmol/L Tris, pH 7.4, 5 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L EGTA (pH 8), 1 mmol/L DTT, 10 μg/mL aprotinin, 10 μg/mL Leupeptin , 1 mmol PMSF and 0.2 mmol/L sodium orthovanadate, at 4 °C for 30 minutes using 2 $x10^6$ cells per 100 μL of lysis buffer for the cell lines. Normally using the total cell number at $1x10^7$ cells per sample. After homogenisation (20 strokes with a B-pestle Dounce homogenizer), sample were centrifuged at 2000x g for 5 minutes to remove the nuclei, and centrifuged again at

10,000x g for 10 minutes to obtain heavy membrane fraction (pellet) containig mitochondria. The supernatant was centrifuged with the ultra-centrifige at 100,000x g , 4 °C for 30 minutes to obtain the pure cytoplasmic fraction. The the heavy membrane fraction will be solubilised in 1% Triton X-100, 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 μg/mL aprotinin, 10 μg/mL Leupeptin , 1 mmol PMSF and 0.2 mmol/L sodium orthovanadate. Then 4x loading buffer were added to lysate from both fractions. Then the equal amount of protein measured by BCA assay were loaded in 10% SDS-PAGE gel.

2.8.2.4. *Micro Biochononinic Acid (BCA) Protein Assay*

The commercial assay kit was purchased from PIERCE company (Rockford, USA, cat No. 23235). Briefly, the cell lysate was diluted at 1:10 in 1x PBS, and then kept on ice until use. Meanwhilst the serial dilution of the standard BSA protein stock (2 mg/mlL) was prepared over 0 – 200 ug/mL and kept on ice. The assay solution was prepared by using 50 parts of solution A, 48 patrs of solution B and 2 parts of solution C, and then was also kept on ice until use.

Then 75 μL each diluted lysate sample was added to 3x wells of 96-well microtiter plate (Nunc-Immuno™ Plate, MaxiSorp™ Surface, Nunc Brand Products, Denmark). Seventy-five micro-litre each diluted standard-BSA sample was added to 3x wells of the microtitre plate. The 75 μL prepared assay solution was then added to each well. Then the plate was incubated for 2 hours at 37 °C. Then the plate was moved to read the wavelength absorbance at 562 nm with the microplate reader. The wavelength value of absorbance of the standard BSA will be plotted vs. protein concentration. Then this curve will be used for determine protein concentration for the lysate.

2.9. Statistical methods

Most data presented in this thesis were described as mean ± 95% confidence interval, or mean with SD when ANOVA or two-way ANOVA analysis were applied. The significant was determined by student t-test where $p < 0.05$ was considered to be significant.

Table 2.3. Patient Characteristics

Condition	Value
Patients	F = 9, M = 20
Age	46 ± 14 years
Hb (g%)	8.45 ± 3.2
White Blood Count (x 10^9/ L)	239 ± 181
% Blasts in BM	4.7 ± 3.7
% BCR/ABL positive by FISH	87.4 ± 7.3
IC50 of imatinib to inhibit p-Crkl by Western Blot	0.53 ± 0.22 μM

Table 2.4. Cell lines used in the studies

Cell lines	Fusion/Chromosome	Comments
K562	b3a2/ Ph1chromosome	Commercially purchased from ATCC (The global Bioresource Centre)
KU 812	b3a2/ Ph1 chromosome	
Meg01	b2a2/ Ph1 chromosome	
MOLM-1	b2a2/ Ph1 chromosome	
wt-BCR-ABL/CTLEN	BCR/ABL	Both were kindly provided by Professor Angel Lopez.
CTLEN	-	

Cytotoxic T-lymphocyte-ecotrophic retroviral receptor neomycin (CTLEN) is a derivative of CTLL-2. CTLEN cells were transduced with retroviral constructes for co-expression of the αsubunit together with wt β_C subunit (pRuf-IRES-$\alpha\beta_C$) (Guthridge et al 2004). CTLEN cells were maintained in medium containing RPMI supplemented with 10% FCS, 2 mM L-Glu, antibiotics, 50 μM β-mercaptoethanol and 100 units/mL mouse IL-2 (Guthridge et al., 2004; Le et al., 2000).

The wt-BCR-ABL/ CTLEN is the GM-CSF$\alpha\beta_C$ receptor expressing cell lines which were transduced for the expression of BCR/ABL. This cell line was first generated in the Division of Immunology in the IMVS by a colleague of Professor Angel Lopez. This cells was maintained in the same medium as K562.

Chapter 3.

The responses of CML CD34$^+$ cells to GM-CSF

3.1. *Introduction*

BCR/ABL drives CML cells to proliferate spontaneously due to its autophosphorylation and cytoplasmic localisation, both of which lead to the constitutive phosphorylation of various downstream targets. (Konopka and Witte 1985, Van Etten, *et al* 1989). The cytoplasmic localization arises from the change of the function of the myristoylation domain, which is lost during the translocation event and is usually found at the N-terminus of c-Abl (normal Abl, 1b isoform), due to the abnormal local adhesion at the C-terminal of BCR/ABL resulting from the constitutive tyrosine kinase activity responsible for adhesive changes (Daley, *et al* 1992, Dhut, *et al* 1990, Van Etten, *et al* 1989, Wetzler, *et al* 1993). Although CML cells have functional defects of granulocytes, monocytes, erythrocytes, megakaryocytes, B-lymphocytes and occasionally T-lymphocytes (Faderl, *et al* 1999), excessive myeloid expansion (Sawyers 1999) suggests that BCR/ABL may activate the signals downstream of cytokine receptors involved in myeloid proliferation and differentiation (Bernheim, *et al* 1981, Dameshek 1951, Martin, *et al* 1980, Whang, *et al* 1963). In addition, the specific myeloid expansion suggests that the abnormal myeloid production occurs in committed stem cells rather than very primitive stem cells. This is confirmed by previous studies showing that CML progenitors have earlier cytoplasmic maturation compared with comparable normal progenitors (Clarkson, *et al* 2003). The evidence of more rapid maturation includes higher proportion of type II blasts with non-specific granules, increased expression of CD33 and more rapid loss of CD34 antigen, higher expression of EPO receptors and a heightened response to EPO, Kit-ligand, and GM-CSF as single cytokines together with a reduced requirement for synergistic activation by multiple cytokines (Goto, *et al* 1982, Strife, *et al* 1988).

This does not mean that the genetic abnormality occurs in committed stage stem cells. The multilineage nature of CML, by the finding of the Ph-chromosome in many cell lineages, strongly indicates that the initiation occurs in progenitors (Holyoake, *et al* 2002). Unknown factors cause myeloid lineages to be susceptible for enormous expansion, unlike other lineages. This reason leads to the investigation of the responses of CML progenitors to the cytokines relating to myeloid expansion. The cytokines that are predominantly involved in general myeloid expansion are EPO, IL-3, GM-CSF and G-CSF. CML progenitors may

have special intrinsic factors to induce early commitment of cells and turn the cell function to have easy susceptibility to extrinsic factors such as cytokines. Then cytokines may play role in cell expansion and enable the CML progenitor cells to overcome and survive stressful conditions in hyper-cellular marrow of the disease or during treatment later.

CML $CD34^+$ cells have been reported to have an increase in cell proliferation in response to growth factors (Moore, *et al* 1998). In contrast to the studies on primary CML cells, those examining BCR/ABL expressing cell lines and IL-3 stimulated cells (Sattler and Salgia 1997), and an activation of IL-3 production in some cell lines after their transformation by BCR/ABL (Anderson and Mladenovic 1996, Hariharan, *et al* 1988, Sirard, *et al* 1994) all have suggested a possible explanation for the ability of BCR/ABL to abrogate their factor dependence. In addition, IL-3 still partially activated the growth of the cell lines in some studies. Therefore, such CML cells are not entirely growth factor independent, but have some responsiveness to certain growth factors which presumably play role in concert with the BCR/ABL tyrosine kinase activity. Abnormal myeloid expansion in chronic phase might be another clue to the identity of growth responsible factors in relation to myeloid cell proliferation and differentiation.

GM-CSF is a glycoprotein with an apparent molecular weight of 23,000-28,000 and is produced by several cells including activated T cells, macrophages, and endothelial cells. GM-CSF may play a role in the pathogenesis of several disease conditions, particularly chronic inflammation and leukaemia. This cytokine has been shown to be necessary for the survival and proliferation of some leukaemic cells in vitro. Certain acute myeloid leukaemia and lymphoblastic leukaemia (Freedman, *et al* 1993) have been demonstrated to exhibit the abnormal regulation of malignant cell-growth in response to autocrine or paracrine GM-CSF (Begley, *et al* 1986, Young and Griffin 1986). GM-CSF exerts its effects through binding to its high affinity receptor (Le Beau, *et al* 1986). Two signal transduction pathways that occur after GM-CSF binds to its receptors involve a distinct region of β_C (Sato, *et al* 1993). The first, which leads to induction of c-myc and activation of DNA replication, involves an activation of Janus kinase (JAK2) that is physically associated with β_C (Quelle, *et al* 1994). Regulation of gene expression by JAK2 appears to be mediated by production of a DNA-binding complex containing the signal transducer

and activator of transcription (STAT) protein (Mui, *et al* 1995, Wang, *et al* 1995). The studies on p210- STAT5A(-/-)/STAT5B(-/-)- reconstituted mice showed that those mice developed CML-like myeloproliferative disorder. However, the incidence of CML in p210 STAT5A/STAT5B knockout mice were 4-fold lower than p210-STAT5 wild-type mice because most p210-STAT5 knockout mice died from either B-cell ALL or a B-ALL/CML mix (Sexl, *et al* 2000). The findings suggested that the pathogenesis of CML was definitely STAT5 independent. However, the presence of B-cell ALL in p210 STAT5A/B knockout mice suggested that STAT5 might associate with the myeloid progenitor function rather than lymphoid progenitor function. Additionally, it may be important for a cause of myeloid expansion. The second pathway involves activation of RAS (Satoh, *et al* 1991) and mitogen-activated protein kinases (MAPK) (Okuda, *et al* 1992), with a consequent induction of c-fos and c-jun, which are genes involved in regulation of haematopoietic differentiation (Quelle, *et al* 1994).

Recently, upregulated expression of HLA-DR was found in most CML progenitors (Holyoake, *et al* 2001, Udomsakdi, *et al* 1992, Verfaillie, *et al* 1992). This marker is known to represent an early response to growth factor activation by their predominantly quiescent primitive normal cells (Srour, *et al* 1992). It suggests that the CML progenitor cells may have an earlier response to some growth factors than normal progenitor cells which are at a comparable stage of maturation. In the human body, there are many cytokines produced by both the cells in the bone marrow and in the extramedulary haemopoietic organs. This production of cytokines may involve or enhance the expansion of CML cells and help CML progenitors to escape spontaneous apoptosis, the treatment induced apoptosis. This idea may support the finding that BCR/ABL driven cells are able to grow spontaneously in the lack of any cytokines, but in terms of cell expansion the primary CML cells but not the cell lines may need the help of other cytokines.

One of these growth factors mentioned previously is GM-CSF, which could be produced by the cells in the microenvironment. The role of GM-CSF has received little attention with respect to on the growth of CML cells. That is because previous studies found the production of other cytokines rather than GM-CSF by CML $CD34^+$ cells in an autocrine fashion. Although GM-CSF is in the same family group as IL-3 and IL-5 that can activate the signal transduction through the same β_C receptors, it can cross-compete with other

cytokines in the same group to bind to the receptor with better affinity to cells that have multiple cytokine receptors (Woodcock, *et al* 1999). In addition, GM-CSF has also been found at high levels in serum of CML patients whilst IL-3 level could not be detected (el-Ahmady, *et al* 1997, Jonuleit, *et al* 1998, Lajmanovich, *et al* 1993) suggesting that GM-CSF may be important to promote CML progenitor survival or may modify CML progenitor response to treatment.

3.2 *Aims of the study*

The aim of the current study is to investigate the ability of GM-CSF to modulate the proliferation and survival of CML progenitors.

3.2.1. To compare the spontaneous proliferation between normal and CML $CD34^+$ cells using the CFSE dye dilution technique

3.2.2. To compare the responses of normal and CML $CD34^+$ cells to GM-CSF as a single cytokine

3.3. *Special Methods*

3.3.1. *Dye dilution technique (CFSE)*

The experimental approach predominantly used was dilution of CFDA-SE (**C**arboxy**f**luorescein **D**iacetate **S**uccinimidyl **E**ster) or CFSE to track the cell proliferation in response of cytokines GM-CSF stimulation. The aims were to compare the effect of the cytokine on the proliferation of CML and normal $CD34^+$ cells particularly the cytokines in the IL-3 superfamily for the reason of the stimulation of the same pathway as BCR/ABL. The proliferation activity is determined in terms of PI (Proliferation Index) calculated using the formula shown later in this chapter.

Fresh or frozen CML or normal cells were used. The cells were ficolled or thawed as described in the general methods in Chapter 2. In the low-percent $CD34^+$ cell sample, cells were first sorted using CD34-MACS kit as described in Chapter 2, and then were

incubated with 10 μM CFSE for the cell number up to 5 x 10^7 cells/mL for 10 minutes in a 37°C waterbath. Then cells were washed thrice with cold sterile 5% FCS in PBS. After the third wash, the labeled cells were resuspended with SDM and incubated in a humidified incubator setting at 37°C and 5% CO_2 overnight. The next day, cells were washed thrice with cold 5%FCS/PBS, and stained with 1 μg of PE-conjugated CD34 and 7-AAD per up to 1 x 10^6 cells. Then labeled cells were incubated for 15 minutes at room temperature before washing twice with cold 5%FCS/PBS. Then the cell pellet was resuspended with 1% BSA/IMDM.

Compensation controls comprised unlabeled cells, CFSE only, CD34- PE only, and ethanol permeabilised cells stained with 7-AAD. Briefly, for the compensation control for 7-AAD, cells were first permeabilized by adding 2-ml 70% ethanol to the cell pellet and keeping on ice for 10 minutes. Then the permeabilized cells were washed twice with 1mL cold 5%FCS/PBS and resuspended with 50μL 5%FCS/PBS containing 1 μg 7-AAD and incubated for 15 minutes at room temperature The cells were washed twice with cold 5%FCS/PBS after incubation, and resuspended with 3 mL of 1% BSA/IMDM.

For culture, 5 x10^4 to 2 x 10^5 cells /mL of sorted cells were seeded in 96-well or 24-well plates. The cell concentrations were appropriate for a short term assay of this type, determined by titration of cell number. The labeled cells were cultured in SDM (**S**erum **D**eprived **M**edium) with or without GM-CSF or other treatments as indicated for 3 days. At the same time, the non-stained/non CFSE cells and CFSE only labeled cells which were left after sorting were cultured separately for use as the compensation controls when evaluating the test cell cultures by flow-cytometry on day 3.

3.3.1.1. *Non-division related decreased in CFSE intensity still allows examination of proliferation between days 2 to 6.*

After labelling cells with CFSE, the labelled cells were tested for the decreased intensity of CFSE during cultures on time course. Briefly, CML $CD34^+$ cells derived from 4 patients in chronic phase were labeled with 10 μM CFSE and cultured overnight. Then, the CML $CD34^+$ cells were washed, and then treated with 100 ng/ml colcemid examining non-

Figure 3.1. The decrease of intensity of CFSE in the absence of cell division

CML $CD34^+$ cells from 4 patients were sorted using PE-conjugated CD34, 7-AAD and the dye dilution CFSE. $1X10^4$ cells were seeded in SDM (Serum Deprived Medium) in 96-well plates treated with 100 ng/mL colcemed to prevent cell proliferation. The labeled cells were cultured over 6 days. Cells were harvested every second day to evaluate mean fluorescence intensity. This Figure showed that there is a sharp drop in CFSE intensity in the first 2 days of culture, after which it is stable. The drop of CFSE was most likely due to the cell catabolism, or loss of small molecular weight labelled amines. The slightly drop of CFSE after 2 days means the stabililty of CFSE and amine content in the cell cytoplasm. This help to ensure that CFSE labeled cells could be evaluated between day 2 to day 6 without the interference of the auto-fluorescence which showed dim intensity on FL 1.

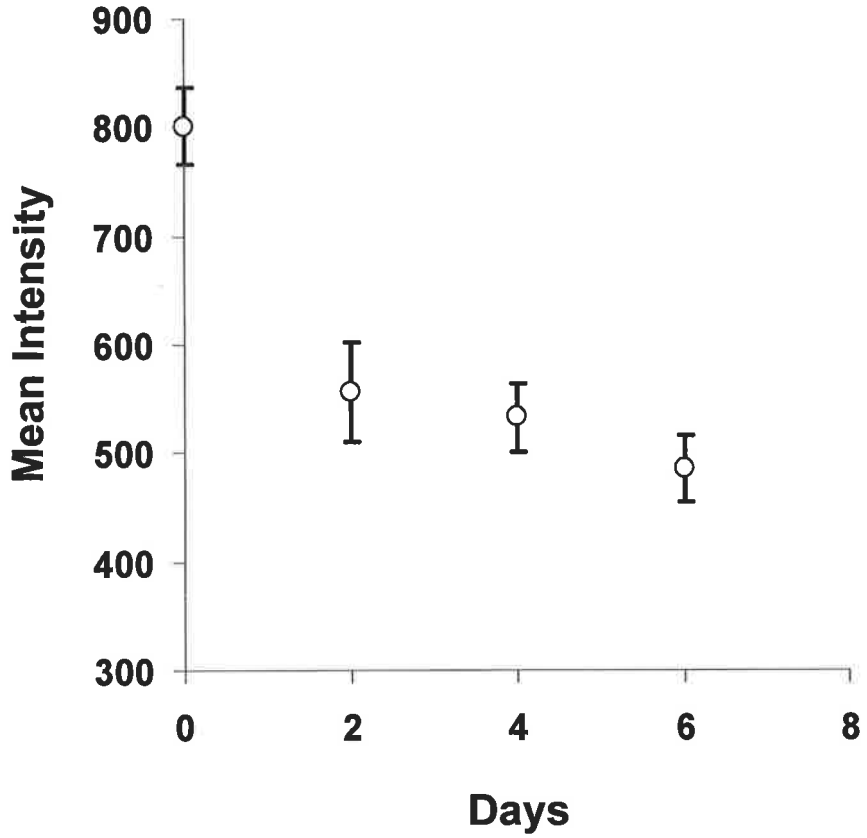


Figure 3.1

division related decreased intensity. After that the cells were harvested every second day for 6 days to analyse the mean intensity of CFSE by flow-cytometry.

There is a decrease of CFSE intensity in the first 2 days, after which the CFSE intensity is quite stable from day 2 to day 6 as shown in Figure 3.1. These time-courses will not be interfered by the autofluorescence of debris and/or the controls without CFSE if the concentration of CFSE is sufficient. Therefore the duration between day 2 and day 6 was suitable for the proliferation studies of the primary CML cell model.

3.3.1.2. *Calculation of proliferation index*

Based on ModFit LT user guide (1994-2000 Verity Software House, Inc. Page 243) and the method to calculate undivided cells from the CFSE dye dilution technique(Lyons and Parish 1994), the proliferation index is defined as the capability of a cell to divide into daughter cells or to proliferate within a fixed duration. To calculate this value, cells should be sorted under narrow ranges of fluorescence to ensure that the input cells in culture have the same CFSE concentration.

The labeled cells that are positive for CD34-PE and negative for 7-AAD were selected under 40 channels of FL-1 which is used for measuring CFSE intensity (Figure 3.2). The result has shown in Figure 3.2 (Panel a and b). This to ensure that once cells have division, the occurring daughter cells in the same division should have the same divided intensity of CFSE which reveal clear division bands on dot-plot diagram Figure 3.2, panel c and d.

In some situations as shown in Figure 3.2, panel e and f, some cells die variable number of cell division. In most PI determinations, the total amount of cell division was calculated from both live and dead CFSE labeled cells. It will be overestimated if the number of cell division and proliferation index are assessed from live cells only. Therefore, 7-AAD is not only able to detect dead cells, but also very useful for making the decision to select the area or gate on flow-cytometry for calculating proliferation index.

Proliferation index is calculated from the sum of absolute number of cells in every division divided by the sum of parent cells that bring about the number of daughter cells in each

Figure 3.2. CFSE dye-dilution method

The primary CML cells were stained with CFSE, and then were incubated overnight at 37°C. Cells then were harvested the next day to be stained with 7-AAD and PE- conjugated CD34 antibody. After staining, cells were sorted to get the $CD34^+$ / $7\text{-}AAD^-$ / $CFSE^+$ cells under 40-channel fluorescence gate on CFSE measurement by $FACStar^{PLUS}$ cell sorter as shown in panel a and b.

Then the sorted cells were cultured in SDM supplemented with imatinib and/or GM-CSF and/or E21R for 3 days. Panel c and d showed the cells on day 0 and day 3 without treatment by histogram which was used to identify the border of each division for evaluation of proliferation index.

Proliferation index (PI) was arisen from the evaluation of total cell division including live and dead cells, not just the division activity of remaining live cells. As shown on panel e and f, sometimes the more division number of dead cells than live cells had been observed. The calculation of PI using the live cells alone might be an over-estimate, thus combining live and dead cells gave a more accurate estimate of division events occurring over the duration of culture.

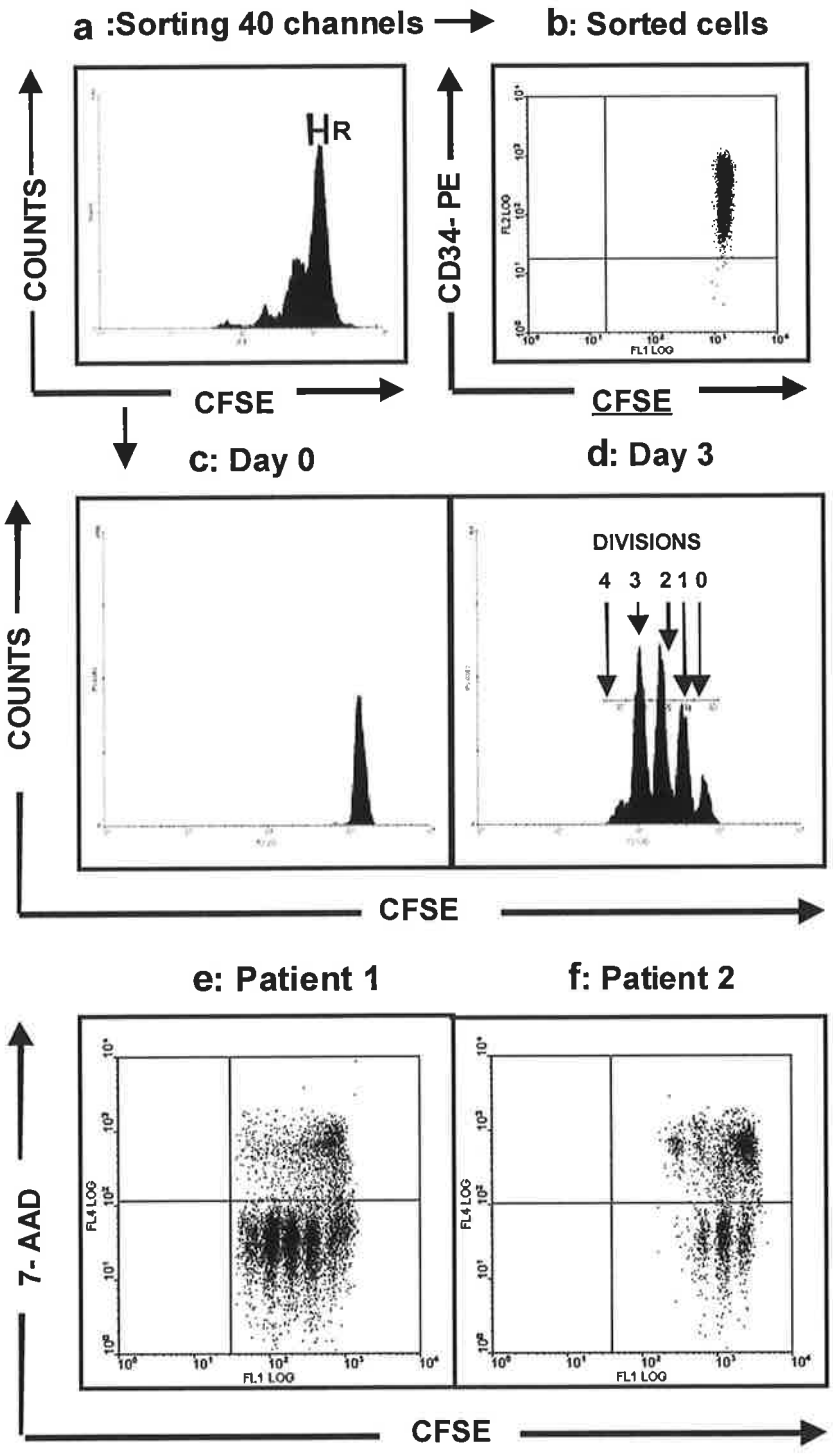

a :Sorting 40 channels
b: Sorted cells
HR
COUNTS
CFSE
CD34- PE
CFSE
c: Day 0
d: Day 3
DIVISIONS
4 3 2 1 0
COUNTS
CFSE
e: Patient 1
f: Patient 2
7- AAD
FL1 LOG
FL4 LOG
CFSE

Figure 3.2

Figure 3.3. The calculation of proliferation index

After 3 days culture in SDM only, CML $CD34^+$ cells showed the characteristic CFSE peaks associated with cell division. Markers set over each discrete peak allowed the total number of events in each to be enumerated. This data together with the formula allowed the proliferation index to be calculated, as shown in Table 3.1 and the text section respectively.

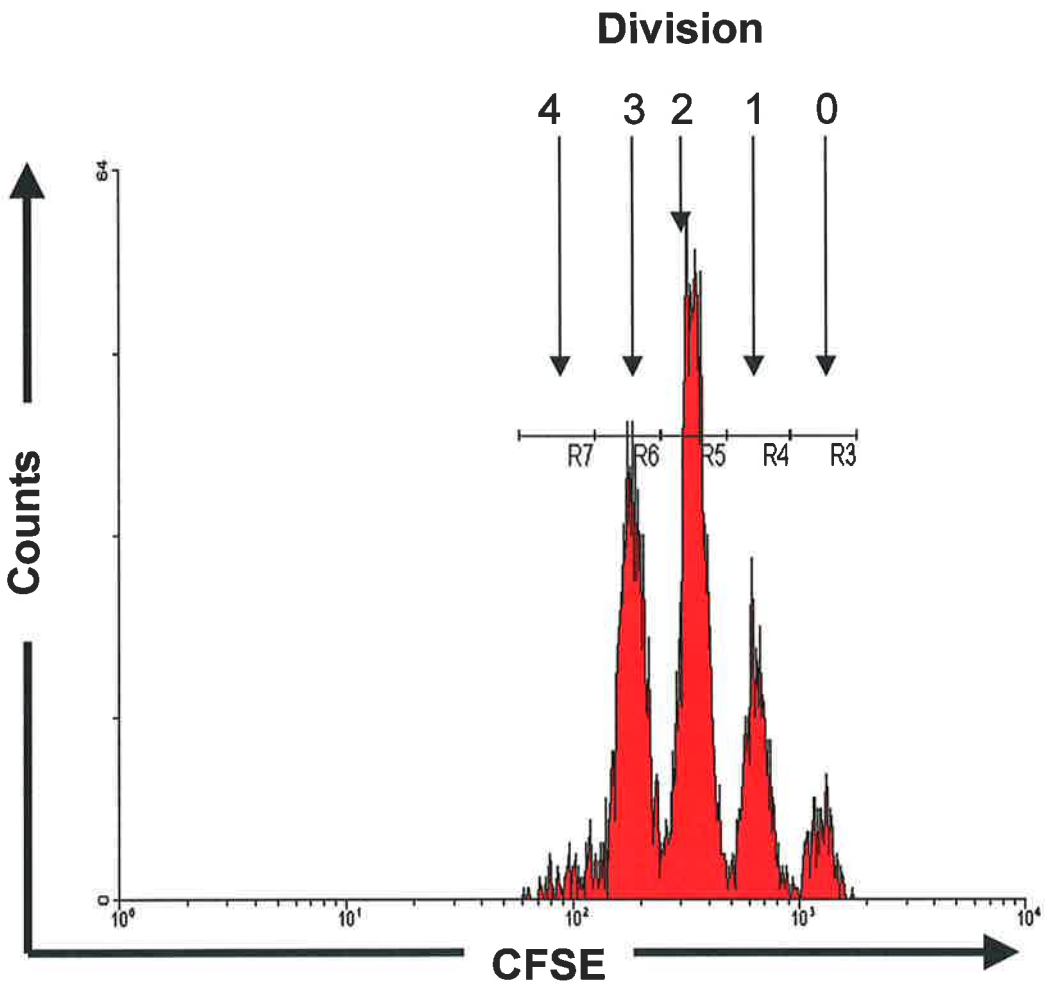

Division
4
3
2
1
0
R7
R6
R5
R4
R3
Counts
64
0
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 3.3

Table 3.1. Using Proliferation Index (PI)
Calculation
Data From Figure 3.3

Division	Events	*f	†Parents
0	561	**1(2^0)**	561
1	1158	**2(2^1)**	579
2	2311	**4(2^2)**	578
3	1541	**8(2^3)**	193
4	142	**16(2^4)**	9
Sum	**5713**	-	**1920**

‡ **PI = 5713/1920 = 2.98**

* *f* = Division Factor

† Parents = Number of parent cells that give rise to the number of events in that division

= Number of Events/ Division Factor *(f)*

‡ PI = Proliferation Index

= The sum of the cells in all generations divided by the calculated number of original parent cells

division as shown in Figure 3.3 and table 3.1. This is based on the knowledge that a parental cell will divide into 2 daughter cells in division 1, 4 cells in division 2, 8 cells in division 3 and $2^{(n-1)}$ cells in division n-1 where n is the number of peaks. The number $2^{(n-1)}$ is called the division factor and is used for the calculation of the number of parent cells that give rise to the number of daughter cells in each division. The number of parent cells at that peak (n) is the result of dividing the absolute number of cells at peak n by $2^{(n-1)}$. Therefore, the formula of the proliferation index (PI) is shown below.

$$PI = \frac{N_1 + N_2 + N_3 + N_4 + \ldots\ldots\ldots\ldots + N_{n-1} + N_n}{N_1/2^0 + N_2/2^1 + N_3/2^2 + N_4/2^3 + \ldots\ldots\ldots\ldots + N_{n-1}/2^{n-2} + N_n/2^{n-1}}$$

The meaning of proliferation index is the fold increasing number compared with the input cells or parent cells. For example, if the PI of cells = 3, therefore the total cell number is 3 fold of the input cells.

3.4. *Results*

3.4.1. *Titration of GM-CSF proliferative activity on CML CD34$^+$ cells*

The proliferative activity of CML CD34$^+$ cells from a patient in chronic phase in the presence of GM-CSF alone at 0-30,000 pg/ml with or without 10 μg/mL E21R, the specific GM-CSFα receptor antagonist has been shown in Figure 3.4. Sorted CML CD34$^+$ were cultured in serum-deprived medium for 3 days before being harvested for flow analysis. E21R was used to ensure that the proliferation peaks occurring in culture with GM-CSF arise from the effect of GM-CSF. With GM-CSF blockade, the proliferation profile should be similar to the culture without GM-CSF.

CML CD34$^+$ cells cultured with GM-CSF only and GM-CSF with E21R were compared (Figure 3.4). The cells with GM-CSF only did not change their proliferation activity compared with those without GM-CSF until GM-CSF > 10 pg/ml which was the concentration for CML CD34$^+$ cells starting to have higher proliferation than the cells in cultures without GM-CSF (The added E21R also aims to imitate the cultures without GM-CSF at each point of GM-CSF concentration and to ensure that the concentration of E21R was sufficient to inhibit the activity of GM-CSF at any level). The proliferation activity

Figure 3.4. GM-CSF titration technique to examine the response of CML $CD34^+$ cells to GM-CSF *in vitro*

By using CFSE dye dilution technique, the optimal concentration of GM-CSF for CML $CD34^+$ cell stimulation in culture was determined by titration of the doses of GM-CSF from 0 to 30,000 pg/ml. We used 10 μg/ml E21R, a specific GM-CSF α receptor antagonist, to confirm that the higher proliferation peaks arose from the effect of GM-CSF. E21R could completely block the activity of GM-CSF receptor until the concentration of GM-CSF was greater than 5,000 pg/ml. Gate R2 in the Figure is the marker for the first peak determined by the overlapping peak of the colcemid treated cells (red peak in division 0).

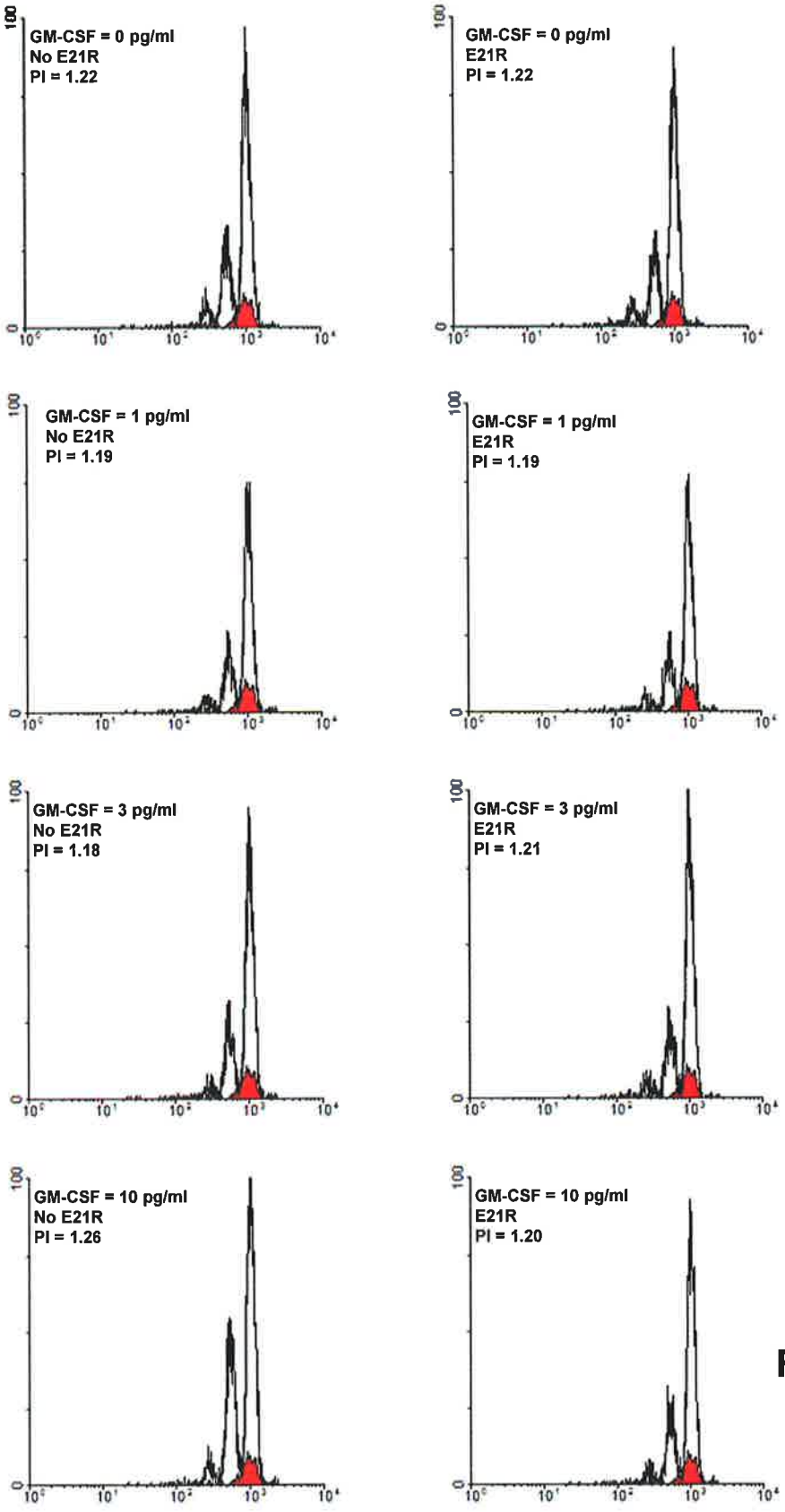

GM-CSF = 0 pg/ml
No E21R
PI = 1.22
GM-CSF = 0 pg/ml
E21R
PI = 1.22
GM-CSF = 1 pg/ml
No E21R
PI = 1.19
GM-CSF = 1 pg/ml
E21R
PI = 1.19
GM-CSF = 3 pg/ml
No E21R
PI = 1.18
GM-CSF = 3 pg/ml
E21R
PI = 1.21
GM-CSF = 10 pg/ml
No E21R
PI = 1.26
GM-CSF = 10 pg/ml
E21R
PI = 1.20

Figure 3.4.1

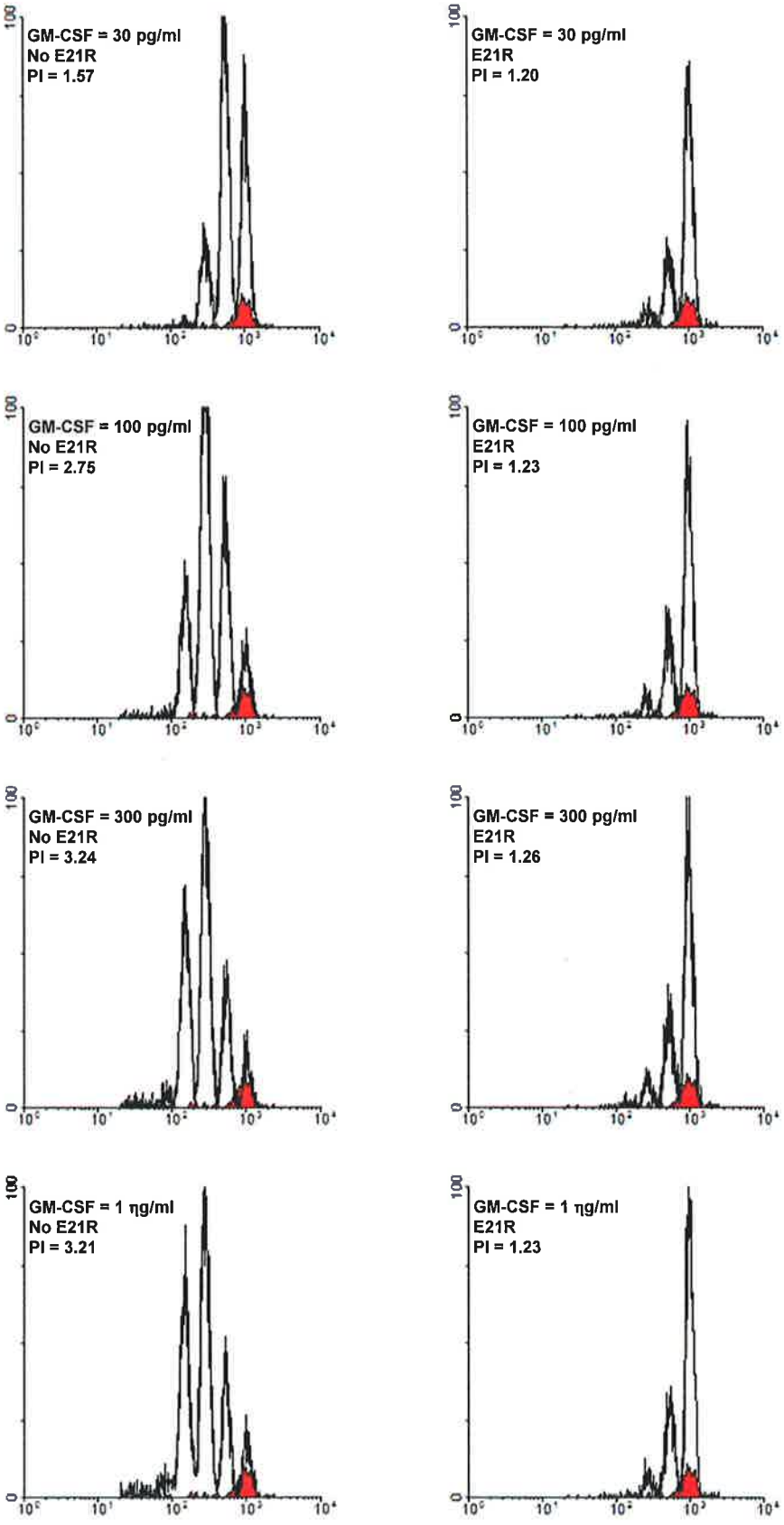
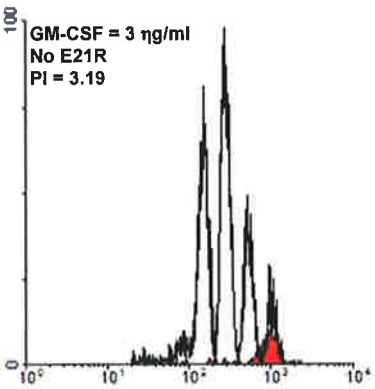
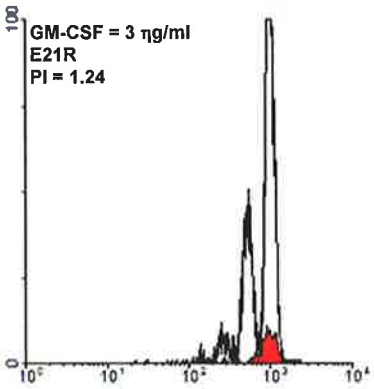
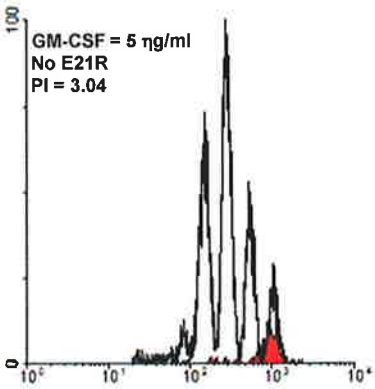
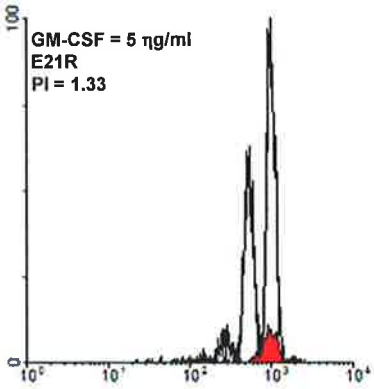

GM-CSF = 30 pg/ml
No E21R
PI = 1.57
GM-CSF = 30 pg/ml
E21R
PI = 1.20
GM-CSF = 100 pg/ml
No E21R
PI = 2.75
GM-CSF = 100 pg/ml
E21R
PI = 1.23
GM-CSF = 300 pg/ml
No E21R
PI = 3.24
GM-CSF = 300 pg/ml
E21R
PI = 1.26
GM-CSF = 1 ηg/ml
No E21R
PI = 3.21
GM-CSF = 1 ηg/ml
E21R
PI = 1.23

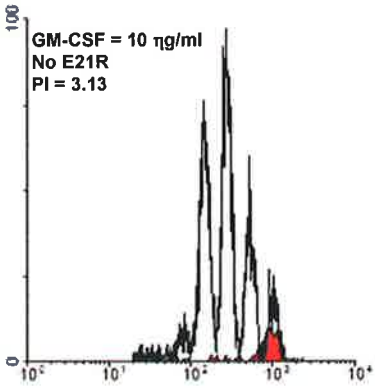
Figure 3.4.2

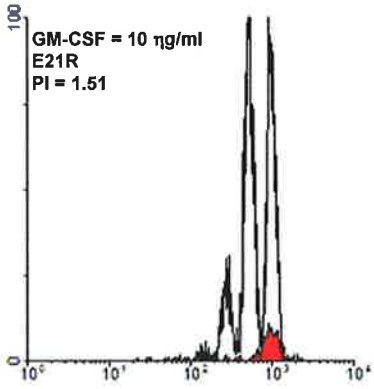

GM-CSF = 3 ηg/ml
No E21R
PI = 3.19

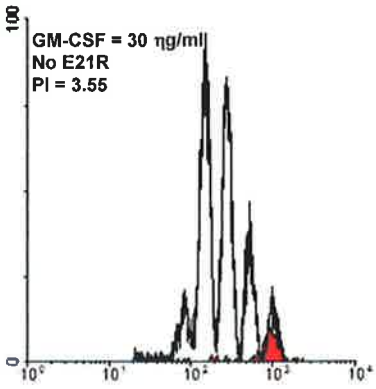

GM-CSF = 3 ηg/ml
E21R
PI = 1.24


GM-CSF = 5 ηg/ml
No E21R
PI = 3.04


GM-CSF = 5 ηg/ml
E21R
PI = 1.33


GM-CSF = 10 ηg/ml
No E21R
PI = 3.13


GM-CSF = 10 ηg/ml
E21R
PI = 1.51


GM-CSF = 30 ηg/ml
No E21R
PI = 3.55

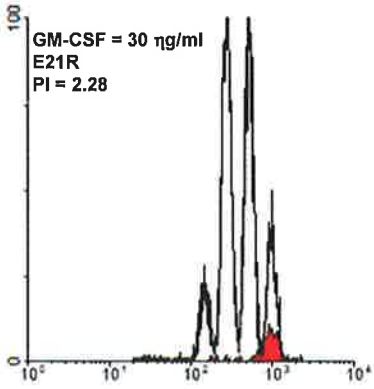

GM-CSF = 30 ηg/ml
E21R
PI = 2.28

Figure 3.4.3

seems plateau at GM-CSF ≥ 300 pg/ml before slightly going up at GM-CSF > 10,000 pg/ml. E21R is quite effective to inhibit the proliferation activity compared with those without GM-CSF. There is no doubt that CML $CD34^+$ cells contain cell surface receptors for GM-CSF as seen from an increase of proliferation activity of CML $CD34^+$ cells following an increase of GM-CSF concentration .

The response to GM-CSF of CML $CD34^+$ cells from 4 patients in chronic phase during 3 days of culture was examined to determine the concentration of GM-CSF giving rise to maximal enhancement of proliferation, as shown in Figure 3.5 and Table 3.2, CML 1-4. CML $CD34^+$ cells were still found that the cell proliferation overrode their basal proliferation when the doses of GM-CSF was greater than 300 pg/ml compared with no treatment (mean PI ± 95% confidence interval = 2.8 ± 0.52 vs. 1.53 ± 0.38, n = 4 respectively). At GM-CSF greater than 300 pg/mL, the trend of proliferation activity of CML $CD34^+$ cells was plateau. Therefore, 300 pg/ml GM-CSF was used as the minimal concentration able to induce maximal proliferation of CML $CD34^+$ cells.

When examining the proliferation index at GM-CSF 0 pg/ml, it was found that E21R alone did not suppress baseline proliferation driven by BCR/ABL suggesting there was insufficient production of GM-CSF by CML $CD34^+$ cells to additive enhance the proliferation of CML $CD34^+$ cells driven by BCR/ABL.

3.4.2. *Comparison of normal and CML $CD34^+$ cell proliferation*

Normal donor cells labeled with CFSE were sorted using PE-conjugated CD34 Ab and 7-AAD to obtain $CD34^+$/7-AAD^- cells under the narrow fluorescence gate (40 channels). 10^4 sorted cells were seeded in 200 mL SDM (**S**erum **D**eprived **M**edium). After 3 days culture in 5% CO_2, humidified incubator at 37^o C, the cells were harvested to evaluate their proliferation by flow-cytometry. The proliferation of normal $CD34^+$ cells was shown in Figures 3.6 without GM-CSF and 3.7 with 300 pg/mL GM-CSF

The CML $CD34^+$ cells have been cultured as the normal $CD34^+$ cells using the same cell number. The proliferation of them was shown in Figures 3.8 without GM-CSF and 3.9 with GM-CSF.

Figure 3.5. GM-CSF titration effects on proliferation of CML $CD34^+$ cells from 4 patients

The proliferation activity of CML $CD34^+$ cells of 4 patients in chronic phase was analysed to examine if the effect of GM-CSF was general. The different patients' cells were found to have subtly different responses to GM-CSF. However, the trend of responses to GM-CSF were quite similar among those patients, which was shown by the increase of proliferation index curve when doses of GM-CSF were increased. The finding that 300 pg/mL GM-CSF was able to stimulate optimal proliferation was confirmed

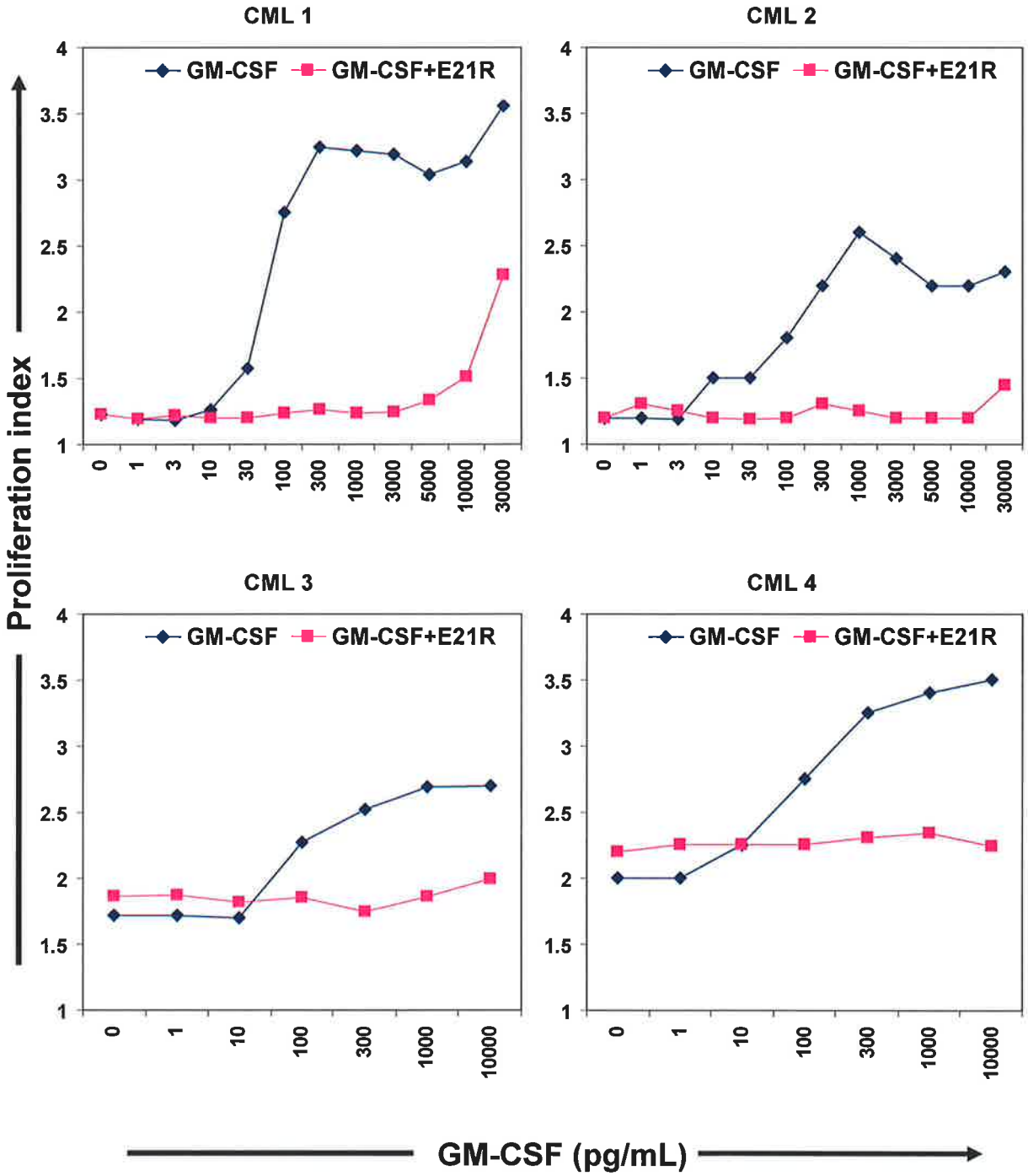

CML 1
CML 2
CML 3
CML 4
GM-CSF
GM-CSF+E21R
Proliferation index
GM-CSF (pg/mL)
0
1
3
10
30
100
300
1000
3000
5000
10000
30000

Figure 3.5

Table 3.2. The data on proliferation index under GM-CSF titration

The Pt1-Pt4 is the patients number 1 – number 4. Mean is mean proliferation index from 4 patients. SD is the calculated standard deviation. 95% CI is the calculated confidence interval.

We can see the different proliferation activity from 4 patients even in the same condition culture. This leads to the high ranges of SD. and 95% CI. However, There is still the similarity of the trend of responses of individual patient, which we assess the appropriate dose of GM-CSF for CML CD34+ cell culture.

Table 3.2. The proliferation index by CFSE dye dilution technique of CML CD34+ cells in the presence of GM-CSF and GM-CSF with 10 μg/ml E21R

	GM-CSF (pg/ml)	Pt1	Pt2	Pt3	Pt4	Mean	SD	95% CI
GM alone	0	1.2	1.22	1.71	2	1.5325	0.390843	0.383019
	1	1.2	1.19	1.71	2	1.525	0.399041	0.391052
	3	1.19	1.18	nil	nil	1.185	0.007071	0.0098
	10	1.5	1.26	1.7	2.25	1.6775	0.42193	0.413484
	30	1.5	1.57	nil	nil	1.535	0.049497	0.068599
	100	1.8	2.75	2.27	2.75	2.3925	0.45522	0.446107
	300	2.2	3.24	2.52	3.25	2.8025	0.527407	0.516849
	1000	2.6	3.21	2.69	3.4	2.975	0.390598	0.382779
	3000	2.4	3.19	nil	nil	2.795	0.558614	0.774185
	5000	2.2	3.04	nil	nil	2.62	0.59397	0.823184
	10000	2.2	3.13	2.7	3.5	2.8825	0.56026	0.549045
	30000	2.3	3.55	nil	nil	2.925	0.883883	1.224977
GM+E21R	0	1.2	1.22	1.86	2.2	1.62	0.493423	0.483546
	1	1.3	1.19	1.87	2.25	1.6525	0.497485	0.487526
	3	1.25	1.21	nil	nil	1.23	0.028284	0.039199
	10	1.2	1.2	1.81	2.25	1.615	0.511762	0.501517
	30	1.19	1.2	nil	nil	1.195	0.007071	0.0098
	100	1.2	1.23	1.85	2.25	1.6325	0.509141	0.498949
	300	1.3	1.26	1.74	2.3	1.65	0.484837	0.475131
	1000	1.25	1.23	1.86	2.34	1.67	0.533854	0.523167
	3000	1.2	1.24	nil	nil	1.22	0.028284	0.039199
	5000	1.2	1.33	nil	nil	1.265	0.091924	0.127398
	10000	1.2	1.51	1.99	2.24	1.735	0.467939	0.458571
	30000	1.45	2.28	nil	nil	1.865	0.586899	0.813385

The normal $CD34^+$ cells had no obvious proliferation in SDM without stimulation. Most cells were dead within 3 days which were shown by the green label in panel b and d in Figure 3.6. Most CFSE labeled cells were $CD34^+$ both live and dead cells as shown in panel c. Therefore the cells were not active in the serum starved condition. In contrast to normal $CD34^+$ cells, CML $CD34^+$ cells were able to divide even in the serum deprived medium with very few dead cells were observed. Some dead cells were not 7-AAD positive, which could be detected through the FS and SS in panel b, Figure 3.8. Those cells have not started to divide as shown in panel d, Figure 3.8- the dots in blue label which were 7-AAD negative, and they had small cell size with huge granulation that could be detect by FS vs SS in blue label.

Adding GM-CSF to culture of normal $CD34^+$ cells did not change the feature of cell activity as shown in Figure 3.7, although more survived and divided cells were observed as shown in panel d. However, added GM-CSF could additively enhance the proliferation of CML $CD34^+$ cells as shown in panel d of Figure 3.9 compared with panel d, Figure 3.8. Dead cells without 7-AAD staining in the division 0 were also found in CML $CD34^+$ cells treated with GM-CSF.

3.4.3. *GM-CSF enhances proliferation activity of CML CD34+ cells*

Spontaneous proliferation of CML $CD34^+$ cells without added cytokines were observed, as shown in Figure 3.10 and table 3.3. When non-treated CML $CD34^+$ cells of 9 patients in chronic phase were evaluated, their mean PI was significantly higher than normal $CD34^+$ cells from 5 normal donors (1.43 ± 0.18 vs. 1.07 ± 0.064 respectively, $p = 0.005$). Interestingly, when 300 pg/ml GM-CSF was added to both CML $CD34^+$ and normal $CD34^+$ cells cultured in SDM, it significantly increased the proliferation activity of the GM-CSF treated CML $CD34^+$ cells over the non-GM-CSF treated CML $CD34^+$ cells (2.29 ± 0.4 vs. 1.43 ± 0.18 respectively, $p = 0.003$). However, there was no significant difference in proliferation index between normal $CD34^+$ cells with and without GM-CSF (1.11 ± 0.031 vs. 1.07 ± 0.064, $p = 0.33$).

3.4.4. *GM-CSF and IL-3 but G-CSF enhance the spontaneous CML $CD34^+$ cell proliferation driven by BCR/ABL.*

Figure 3.6. Normal $CD34^+$ cells showed very little proliferation in SDM only culture.

Normal BM $CD34^+$ cells were sorted to get $CD34^+$ / 7-AAD^- cells within a 40-channel gate of CFSE- intensity. Cells were seeded in SDM without treatment for 3 days in humidified incubator at 37 °C and 5% CO_2. The cells were harvested to evaluate the proliferation activity. Flow-cytometry showed most cells died during culture with little division. Very few of $CD34^+$ cells (15%) survived without stimulation which was confirmed by the 7-AAD stain and FS and SS. This experiment showed that surviving normal $CD34^+$ had no spontaneous proliferation. The panels a, b, c, and d represented the cells from the same sample that were analysed in different colours detected by flow cytometry. The blue colour dots represent live cells which are negative for 7-AAD, and the green are dead cells which are positive for 7-AAD.

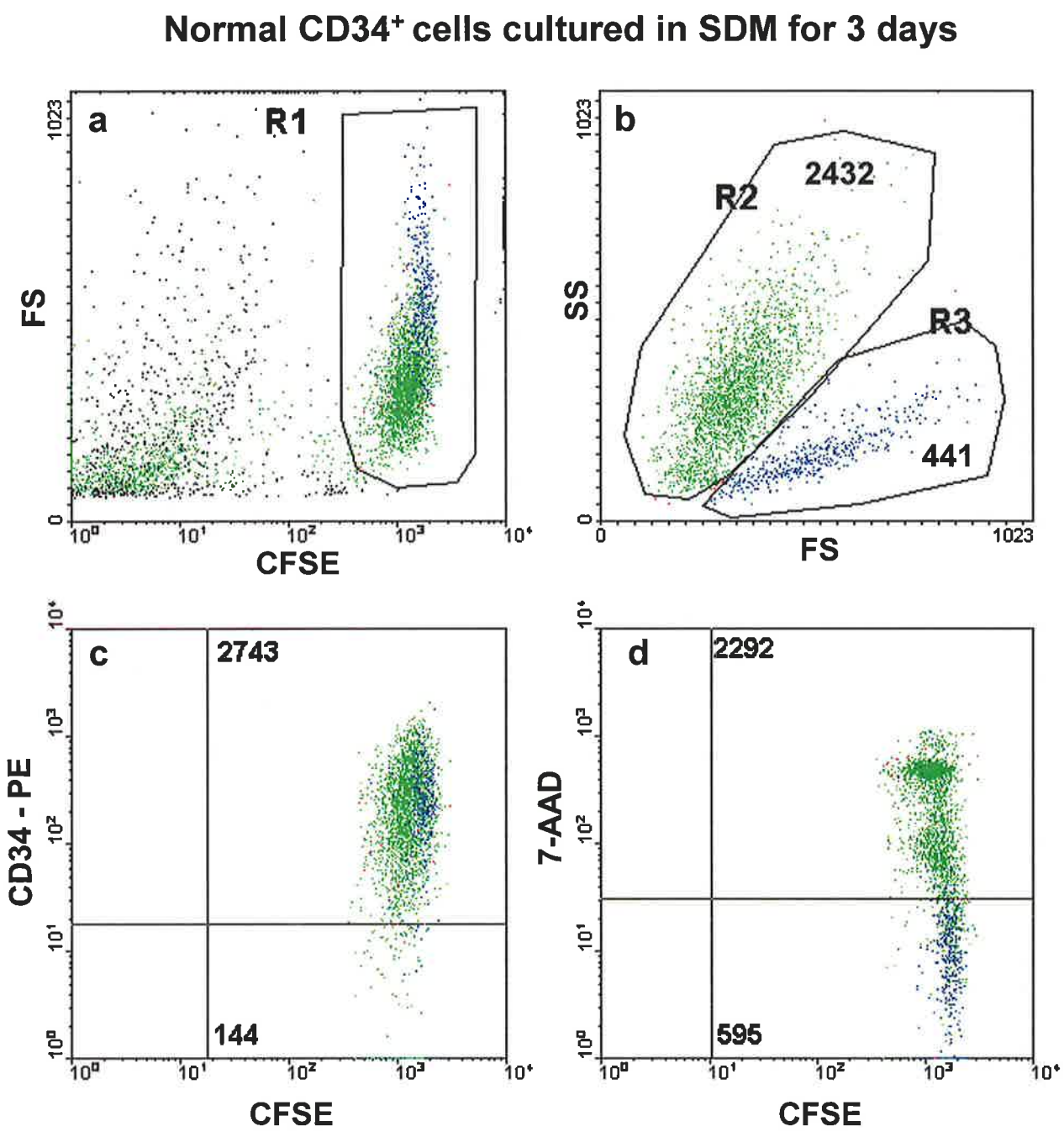

Normal CD34+ cells cultured in SDM for 3 days
a
R1
FS
CFSE
b
R2
2432
R3
441
SS
FS
c
2743
144
CD34 - PE
CFSE
d
2292
595
7-AAD
CFSE

Figure 3.6

Figure 3.7. Normal $CD34^+$ cells in SDM with GM-CSF still showed little proliferation but had improved viability.

Sorted normal $CD34^+$ cells were seeded in SDM supplemented with 300 pg/mL GM-CSF. Although there were still a lot of dead cells, viability was improved to 24% and there was a small amount of proliferation observed. (The blue color dots represent live cells which are negative for 7-AAD, and the green are dead cells which are positive for 7-AAD.)

Normal CD34^{+} cells cultured with GM-CSF for 3 days

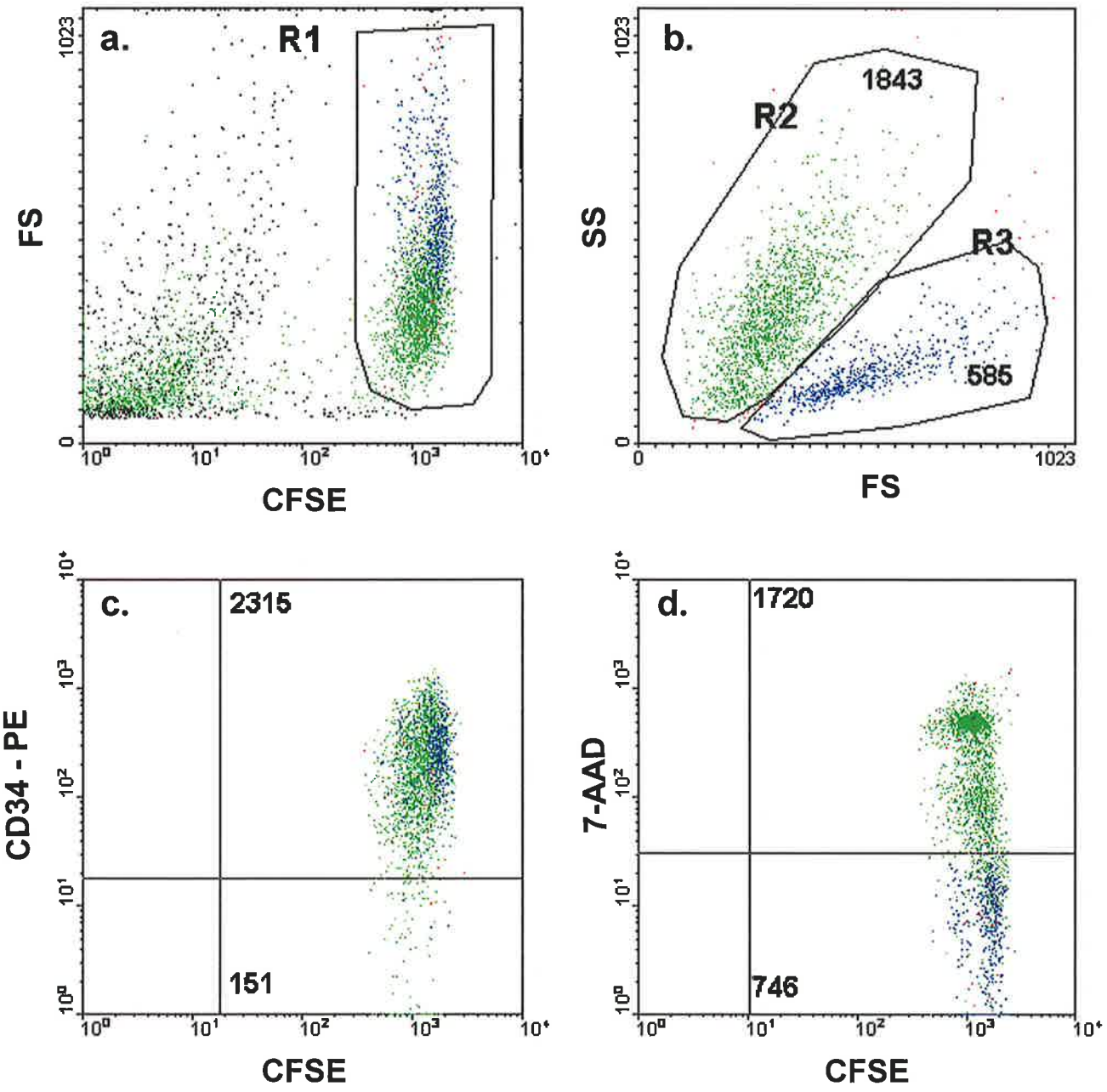


Figure 3.7

Figure 3.8. CML CD34$^+$ cells showed spontaneous proliferation in SDM.

Sorted CFSE labeled CML CD34$^+$ cells were cultured in SDM only for 3 days The cells showed high level of proliferation compared with the normal CD34$^+$ cells in SDM only (Figure 3.6). CML CD34$^+$ cells underwent up to 3 division cycles. Viability was ~ 70% while was markedly higher than normal CD34$^+$ cells in SDM. (The green colour dots represent live cells which are negative for 7-AAD, and the blue are dead cells which are positive for 7-AAD.)

CML CD34+ cells cultured in SDM for 3 days

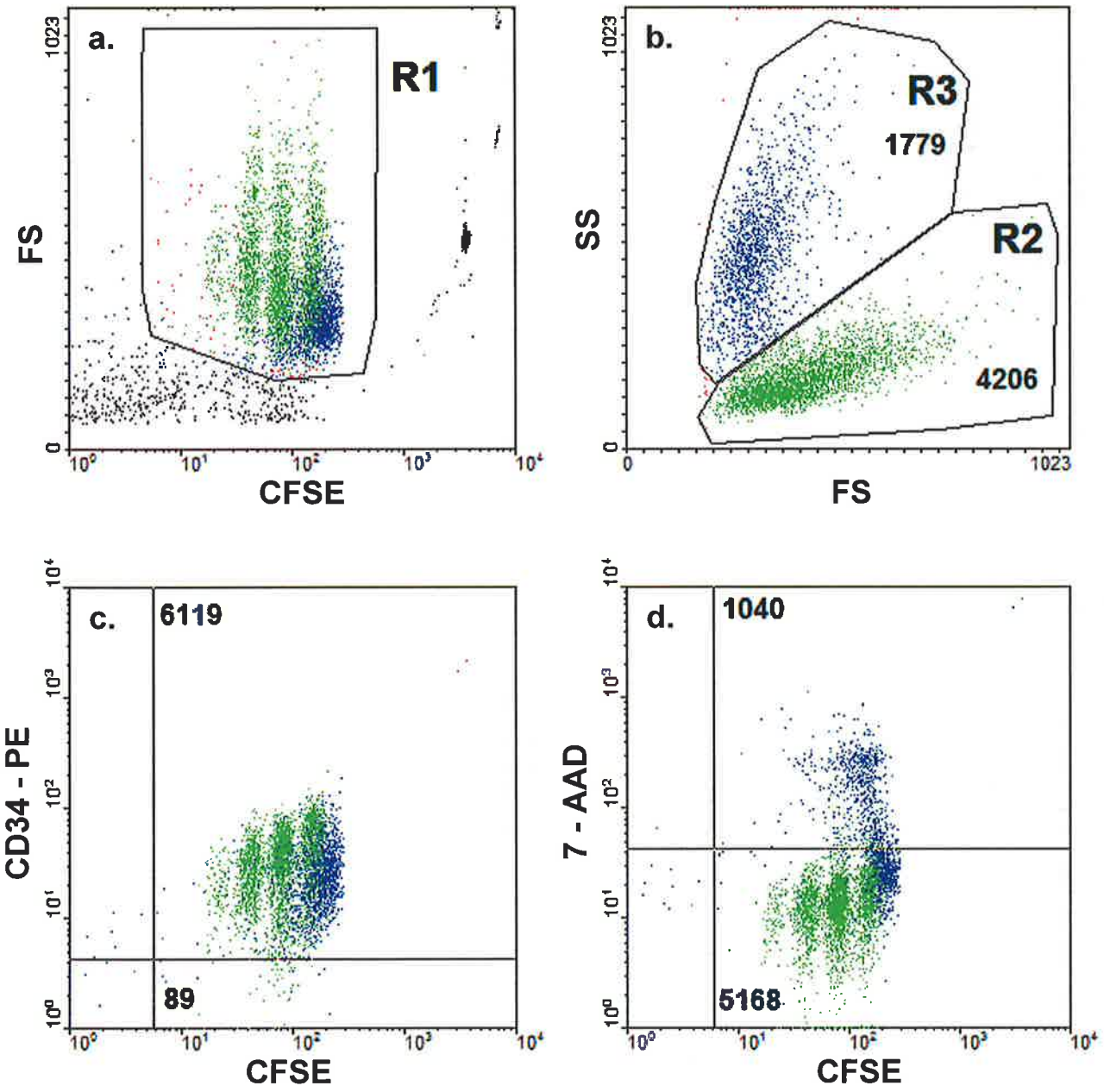


Figure 3.8

Figure 3.9. CML CD34$^+$ cells showed marked increase of cell division in the presence of GM-CSF.

The CML CD34$^+$ cells cultured in SDM with 300 pg/mL GM-CSF in this figure showed the cells had enhanced division with more events in further division cycles compared with the cells in SDM only culture. GM-CSF also improve the viability of the cells in SDM up to 85%. (The green colour dots represent live cells which are negative for 7-AAD, and the blue are dead cells which are positive for 7-AAD.)

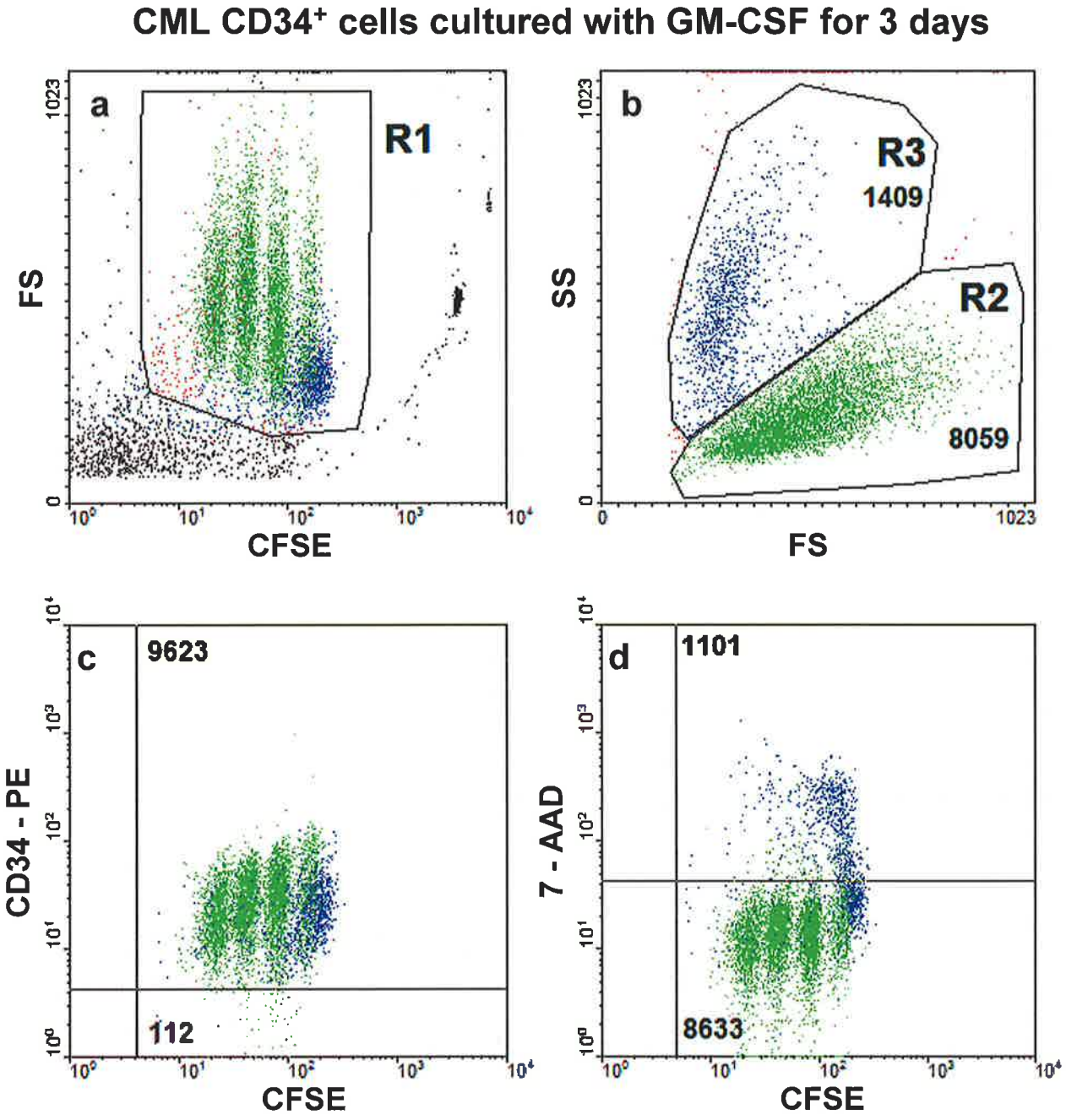

CML CD34+ cells cultured with GM-CSF for 3 days
a
R1
FS
CFSE
b
R3
1409
R2
8059
SS
FS
c
9623
112
CD34 - PE
CFSE
d
1101
8633
7 - AAD
CFSE

Figure 3.9

Figure 3.10. Comparison of spontaneous and GM-CSF driven proliferation of CML $CD34^+$ and normal $CD34^+$ cells.

CFSE labeled CML and normal $CD34^+$ cells from 9 patients and 5 normal donors were cultured in SDM with or without 300 pg/ml GM-CSF. After 3 days, the proliferation index was calculated. CML $CD34^+$ cells had a significantly higher mean proliferative index than normal $CD34^+$ cells in SDM alone (PI = 1.43 ± 0.18, n=9 VS. PI = 1.07 ± 0.064 , n=5 respectively, $p< 0.005$). CML $CD34^+$ cells responded to GM-CSF with enhanced proliferation (PI = 2.3 ± 0.4, VS. 1.43 ± 0.18, n = 9, p= 0.003) whereas normal $CD34^+$ cells did not (PI = 1.11 ± 0.031 VS. 1.07 ± 0.064, n = 5, p= 0.326). Student-t test was applied, where $p < 0.05$ was considered to be significant.

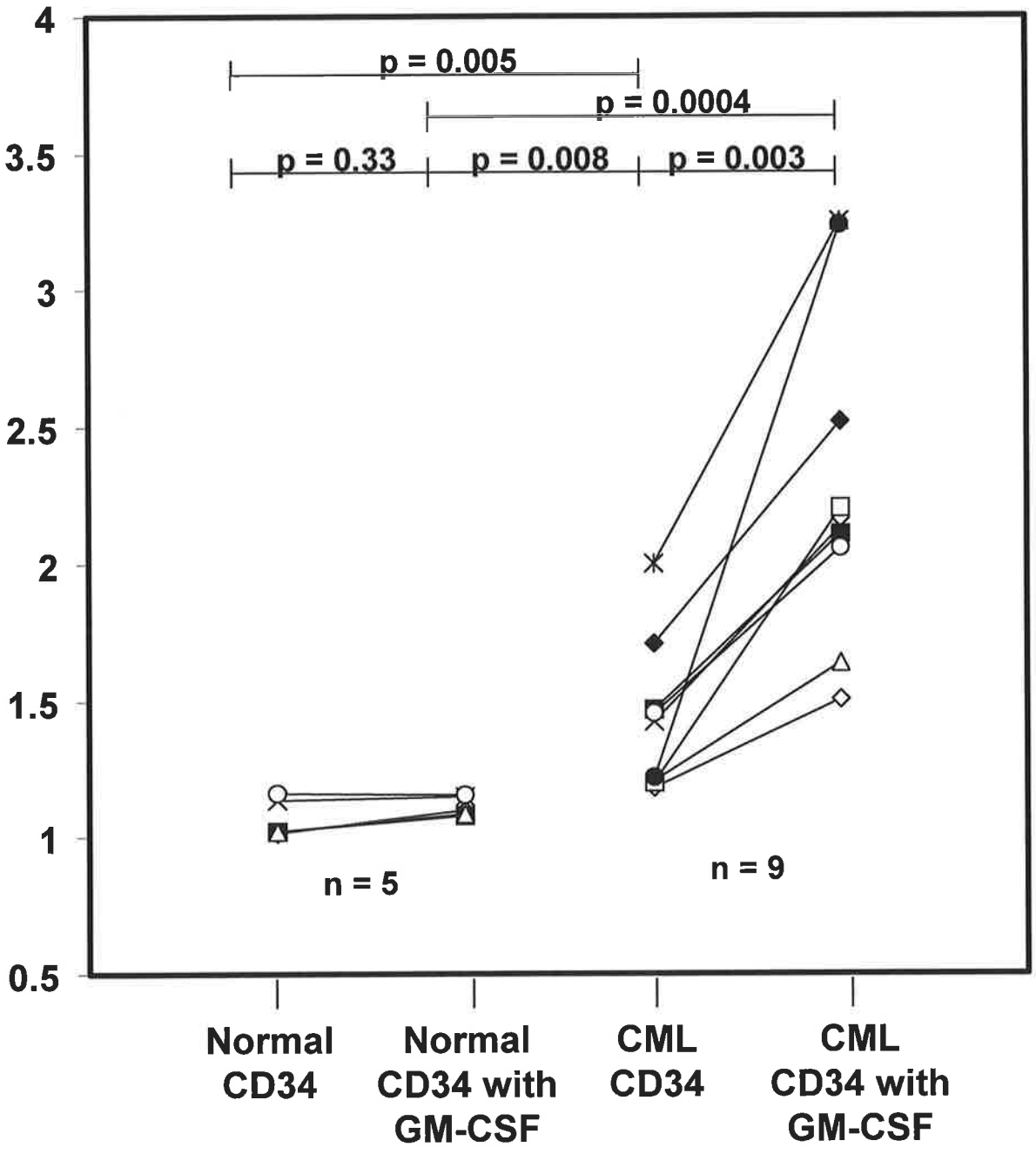

4
3.5
3
2.5
2
1.5
1
0.5
p = 0.005
p = 0.0004
p = 0.33
p = 0.008
p = 0.003
n = 5
n = 9
Normal CD34
Normal CD34 with GM-CSF
CML CD34
CML CD34 with GM-CSF

Figure 3.10

Table 3.3. Proliferation index of normal and CML CD34$^+$ cells

	1	2	3	4	5	6	7	8	9	Mean	SD	95%CI
Nor (no GM)	1.01	1.023	1.018	1.14	1.16					1.071	0.073	0.064
Nor (GM)	1.09	1.077	1.082	1.15	1.15					1.11	0.036	0.031
CML (no GM)	1.18	1.467	1.204	1.42	1.45	1.2	1.22	1.71	2.01	1.429	0.28	0.183
CML (GM)	1.5	2.105	1.635	2.13	2.06	2.2	3.24	2.52	3.25	2.293	0.617	0.403

Nor = Normal CD34$^+$ cells, GM = 300 pg/mLGM-CSF, no GM = Non-GM-CSF culture, CML = CML CD34$^+$ cells

The CML $CD34^+$ cells from three new patients were tested with other cytokines relating to the myeloid proliferation, and their proliferation was compared with the cells in culture with GM-CSF. These CML cells have been tested as well to have an average of 80% FISH-positive for BCR/ABL fusion gene (76%, 84% and 81%). Again cell proliferation assay was done using CFSE dye-dilution technique. The concentrations of GM-CSF, IL-3 and G-CSF used in the experiment all equated 300 pg/mL. The graph shown in Figure 3.11 demonstrated that the effect of all conditions of treatment on CML $CD34^+$ cells was significant overall ($F_{3,8}$ = 18.859, p = 0.001 by ANOVA analysis), and G-CSF at 300 pg/mL (Mean = 2.17, SD = 0.15) could not significantly enhance the proliferation of CML $CD34^+$ cells over their spontaneous proliferation driven by BCR/ABL kinase as shown in CML $CD34^+$ cells without cytokine (Mean = 1.98, SD = 0.36). However, both GM-CSF (Mean = 3.13, SD = 0.24) and IL-3 (Mean = 3.24, SD = 0.23) had similar effect on enhancing cell proliferation of the CML $CD34^+$ cells over their proliferation driven by BCR/ABL kinase and BCR/ABL kinase plus G-CSF. The data has been shown in Table 3.4.

3.5. *Discussion*

G-CSF and IL-3 but not GM-CSF have been reported to be produced by CML $CD34^+$ cells. However, the previous evidence about the high level of GM-CSF in patients' serum rather than G-CSF and IL-3 (el-Ahmady, *et al* 1997, Jonuleit, *et al* 1998, Lajmanovich, *et al* 1993) suggests all of them may be important for CML cell growth and keep CML progenitors latent in bone marrow. The added specific blocking agent E21R did not result in a decrease in baseline CML $CD34^+$ cell proliferation. It suggests that the CML $CD34^+$ cells did not elaborate sufficient GM-CSF into the cultures to enhance proliferation (Figures 3.4 and 3.5). However, other cytokines and the low level of GM-CSF produced by CML $CD34^+$ cells could not be excluded. The low level of cytokines might be important for maintenance of cell survival rather than cell proliferation. Although CML cells have been reported to grow without the need of cytokines, those reports were studied on the BCR/ABL cell lines or the BCR/ABL transfected primary cells which might be different from the primary CML cells (Li, *et al* 2001) (Hariharan, *et al* 1988, Sirard, *et al* 1994).

Figure 3.11. G-CSF had a minor effect on proliferation of CML CD34$^+$ cells compared with GM-CSF and IL-3.

CML CD34$^+$ cells from three patients were sorted and cultured in SDM supplemented with 300 pg/mL each of either G-CSF, IL-3 and GM-CSF. The proliferation index from CFSE dye dilution was calculated to compare proliferation occurring in the three conditions with the the non-treated cells after 3 days of culture. The spontaneous proliferation of CML CD34$^+$ was not very much enhanced by G-CSF. GM-CSF and IL-3 provided quite similar enhancement of the activity of spontaneous proliferation driven by BCR/ABL.

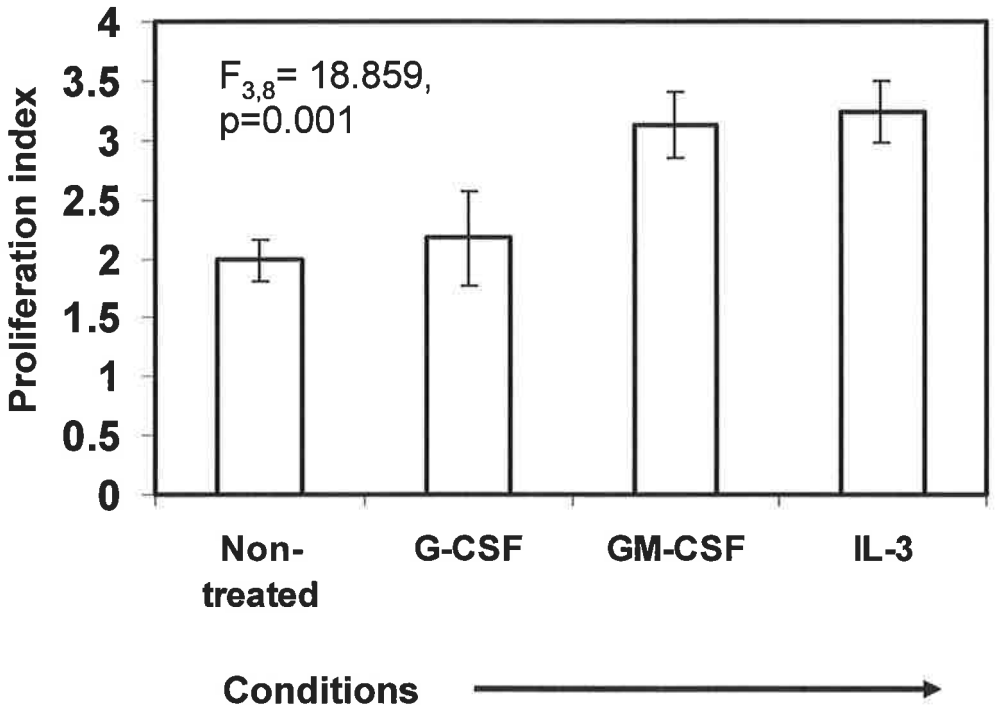

Proliferation index
4
3.5
3
2.5
2
1.5
1
0.5
0
$F_{3,8}$= 18.859,
p=0.001
Non-treated
G-CSF
GM-CSF
IL-3
Conditions

Figure 3.11

Table 3.4. Comparison of proliferation index of CML $CD34^+$ cells in responses to G-CSF, GM-CSF, and IL-3

Proliferation index						
Cytokines	**P1**	**P2**	**P3**	**mean**	**SD**	**95% CI**
No	2.15	1.85	1.95	1.98	0.15	0.17
G-CSF	2.2	1.8	2.51	2.17	0.36	0.40
GM-CSF	2.85	3.3	3.24	3.13	0.24	0.28
IL-3	2.97	3.41	3.33	3.24	0.23	0.27

No = Non-cytokine cultures,
One-way ANOVA analysis showed $F_{3,8} = 18,859$ and $p = 0.001$.

In the titration of GM-CSF, GM-CSF was not found to have any effect on CML $CD34^+$ cells until its dose reached 30-100 pg/ml. Other cytokines were thought to have the threshold of inducing cell proliferation as well. Therefore, the proliferation of CML $CD34^+$ cells should not be enhanced by the low level of other cytokines as well. Another possibility is that the CML $CD34^+$ cells may produce other cytokines at high level as other researchers' observation, but they might have very low level of the specific receptors for other cytokines apart from GM-CSF. However, the receptors for G-CSF and IL-3 have indirectly been shown to be expressed by CML $CD34^+$ cells in Figure 3.11. Therefore, their levels produced by CML $CD34^+$ cells should not be high enough to override the effect of BCR/ABL tyrosine kinase.

An average concentration of 300 pg/ml GM-CSF achieves maximal stimulation of CML $CD34^+$ cells, after which increasing levels resulted in a plateau. The addition of 10 μg/ml of the specific GM-CSF antagonist E21R completely blocked GM-CSF simulation. The level of GM-CSF on the titration experiment has revealed the level of effective concentration of GM-CSF to be used for other experiment.

Normal $CD34^+$ cells showed essentially no spontaneous proliferation and no increase in proliferative response to added GM-CSF. In contrast, CML $CD34^+$ cells showed a higher level of spontaneous proliferation than normal cells, which was markedly enhanced by GM-CSF. The ability of BCR/ABL to drive proliferation in the absence of exogenous cytokines has been previously demonstrated (Eaves, *et al* 1986). The ability of GM-CSF to promote additional proliferative activity to that induced by the tyrosine activity of this oncogene supports the concept that the proliferative activity of CML progenitors are not maximally stimulated by BCR/ABL and retain responsiveness to cytokines. Thus, even though GM-CSF receptor signalling uses some pathways in common with BCR/ABL, it apparently provides additional signals. The finding that CML $CD34^+$ cells were hypersensitive to GM-CSF as a single agent compared with normal $CD34^+$ cells is in contrast with previous reports, however, in those studies, multiple cytokine combinations were used (Bedi, *et al* 1994, Clarkson and Strife 1993, Otsuka, *et al* 1991). Our studies focused on the primary CML $CD34^+$ cells, and examined a single cytokine GM-CSF which was though to utilize a similar signalling pathway to BCR/ABL. Importantly, we

demonstrated that the specific cytokine antagonist E21R was able to completely block the increased proliferation of CML $CD34^+$ cells induced by GM-CSF.

The reason why we tested the response of CML and normal $CD34^+$ cells to GM-CSF is based on previously published experiments on autocrine production of cytokines which suggested the effect of local cytokines including GM-CSF, IL-3 and G-CSF may help in pathogenesis of CML(Hariharan, *et al* 1988, Sirard, *et al* 1994), and the basic knowledge about the signaling pathways downstream from the GM-CSF receptor and BCR/ABL protein. IL-3 have been investigated extensively on both BCR/ABL^+ cell lines and CML $CD34^+$ cells, however, GM-CSF which is in the same group of IL-3 sharing the common β_C receptor has not been widely mentioned. GM-CSF should stimulate cell growth in the same fashion as IL-3 but not G-CSF which had no common receptor portion as seen in IL-3 and GM-CSF receptors. We tested this hypothesis by using the same CFSE dye dilution technique to compare the proliferation activity of CML $CD34^+$ cells in among the cultures with SDM only or SDM with GM-CSF as the single stimulation. Similar to normal $CD34^+$ cells, $CMLCD34^+$ cells express many cell-surface cytokine receptors but not every receptor responds to the binding of its ligand very well. The reason about the different responses of these receptors on the normal $CD34^+$ cells is not clearly understood. We only knew that the proliferation and differentiation of normal $CD34^+$ cells need the combination of multiple cytokines working together. For CML cells, the expression of BCR/ABL may reduce the effect of G-CSF receptor to recruit intracellular tyrosine kinase protein but not the receptors in IL-3 receptor family(Holyoake, *et al* 2001, Zhang and Ren 1998).

The comparison of the ability of cytokines G-CSF, GM-CSF and IL-3 to enhance the activity of BCR/ABL helped to support the finding on the GM-CSF titration assay that the threshold of cyokines might be important for the stimulation of the receptor. GM-CSF and IL-3 had a similar stimulation of the proliferation of CML $CD34^+$ cells. In the titration experiment, if CML $CD34^+$ cells produce IL-3 as found by Jiang X *et al.*(Jiang, *et al* 1999), the level might be very low and not sufficient to promote cells proliferation as found when testing the exogenous GM-CSF, and this was the reason why the added E21R should suppress cell proliferation in the non-treated cells to the lower level than the spontaneous proliferation level.

In short, CFSE dye dilution technique has an advantage over other methods for it covalently binds cytoplasmic macro-molecules with fluorescent emission at 518 nm. and remains stable in cultured cells without affecting their viability. It has a great resolution power to detect a discrete number of divided cells. The combination of CFSE and 7-AAD helps to discriminate dead cells with the CFSE-labeled cells to ensure purified cells in culture. We used all these techniques to determine the differences between CML and normal CD34$^+$ cells in response to GM-CSF as a single cytokine. CML CD34$^+$ cells have been found in this experiment to have spontaneous proliferation without added exogenous cytokines, but this is not quite obviously seen in normal CD34$^+$ cells. They also responded to GM-CSF as a single cytokine much better than normal CD34$^+$ cells. This helps to evaluate the effect of GM-CSF on the modulation of CML CD34$^+$ cells in response to imatinib treatment.

Chapter 4.

GM-CSF modulated the effect of imatinib on CML CD34$^+$ cells

4.1. *Introduction*

Although imatinib is able to profoundly inhibit BCR/ABL tyrosine kinase activity and the clinical studies are very impressive, primary and acquired resistance in some patients have been observed. Mechanisms include BCR/ABL gene amplification, BCR/ABL tyrosine kinase mutation and potentially as yet unknown causes (Branford, *et al* 2002, Druker, *et al* 2001a, Gorre, *et al* 2001, Hofmann, *et al* 2002, Sirulink, *et al* 2001, von Bubnoff, *et al* 2002). Clinical studies with imatinib began in June 1998. Phase I/II trials targeted advanced phase CML, Ph^+ ALL, acute leukaemia (AML), and chronic phase leukaemia refractory to interferon. Concentration of imatinib used at the time ranged from 25 mg/d to 1000 mg/d. At doses ≥ 300 mg/d, 95% chronic phase patients were found to gain complete haematologic responses, and that 60% of the patients had major and 41% complete cytogenetic responses with severe side effects rare (Druker, *et al* 2001a, Druker, *et al* 2001b). With close follow-up, some patients developed molecular resistance to imatinib. CML progenitors may survive imatinib therapy by some unknown mechanisms allowing selective mutation in the common ATP loop, and giving rise to imatinib resistance. Other mechanisms involved in molecular resistance are reduced uptake or increased efflux of drug mediated by P-glycoproteinn and other transport proteins, loss of BCR/ABL protein expression and activation of compensatory survival pathways, and sequestration of drug to α-1 acid glycoprotein (AGP) in body fluid (Azam, *et al* 2003, Gambacorti-Passerini, *et al* 2000, le Coutre, *et al* 2000, Mahon, *et al* 2000, Shah, *et al* 2002, von Bubnoff, *et al* 2002, Weisberg and Griffin 2000). Among the resistant groups, those with primary resistance without a defined mechanism required further investigation. Even in the good response group, BCR/ABL positive progenitors or stem cells are still found in the bone marrow (Hochhaus 2003). Recent reports on many tyrosine kinase inhibitors i.e. PD164199, PD180970 and imatinib on the survival of the BCR/ABL^+ cell lines ($32Dp210^{BCR/ABL}$ cells) have demonstrated that IL-3 could compensate for drug effects (Dorsey, *et al* 2002). Interestingly, the primitive CML $CD34^+$ cells from CML patients were observed to have an autocrine IL-3 loop (Jiang, *et al* 1999). These observations raised the possibility that the survival of the leukaemic clone may result from the protective effect of some cytokines.

The GM-CSF receptor is a member of the IL-3 cytokine receptor gene family and consists of a heterodimer of α- and β-subunits (Blalock, *et al* 1999). The α subunit of this family determines cytokine specificity and has 3 main domains: ligand binding, transmembrane and intracellular signaling domains. The intracellular domain of α-subunit lacks intrinsic kinase activity, but is required for activation of STAT 5, Raf-1 and $p70^{56K}$ (Doyle and Gasson 1998, Tian, *et al* 1996, Watanabe, *et al* 1996).

The β_C (common β) subunit interacts with the relevant α subunit on binding of GM-CSF, IL-3 and IL-5. The β_C subunit also has no intrinsic kinase activity as found in α-subunit (Itoh, *et al* 1998, Kremer, *et al* 1993, Miyajima, *et al* 1993). The gene of this receptor subunit in human is located on chromosome 22q 13.1. The signal transduction through the β_C subunit requires a conformation change on heterodimerisation to promote the binding and activation of downstream targets including JAK, STAT, and PI3K and other molecules such as Shc, which recruited Grb2 and SOS to the Ras/Raf/MEK/ERK cascade (Blalock, *et al* 1999, Miyajima, *et al* 1993).

The initiating event for signal transduction is the binding of GM-CSF, IL-3 and IL-5 to their surface receptors (Hayashida, *et al* 1990, Kitamura, *et al* 1991, Tavernier, *et al* 1991). There was no downstream signal activation without the combination of α and β receptors together. The GM-CSF ligand first binds to its α-specific receptors with low affinity. Then this combination leads to the binding complex with β-receptor with very high affinity. The engagement of β_C chain after binding to the ligand and α chain leads to the tyrosine phosphorylation of the β_C chain and becomes the crucial step to create docking sites for the binding of signaling molecules (Muto, *et al* 1996). Previously, two distinct domains were shown to be responsible for the activation of signal transduction downstream from the β_C chain engagement (Kinoshita, *et al* 1995, Sato, *et al* 1993). These are the membrane proximal domain of the β_C chain accountable for activation of the Jak/STAT and PI3K pathways, and the distal domain accountable for activation of Ras-Raf pathway (Blalock, *et al* 1999, Kinoshita, *et al* 1995, Sato, *et al* 1993).

Over the past few years, BCR/ABL has been shown to stimulate the Ras/Raf/MEK/ERK, Jak/STAT and PI3K/Akt pathways which are responsible for proliferative and anti-

apoptotic effects (Keeshan, *et al* 2002, Kindler, *et al* 2003, Lin, *et al* 2000, Liu, *et al* 1999, Neshat, *et al* 2000, Nieborowska-Skorska, *et al* 2000, Sattler, *et al* 1999, Shuai, *et al* 1996, Skorski, *et al* 1995, Steelman, *et al* 2004). Experimental BCR/ABL transformed cells have been demonstrated to hyper-activate all of these pathways (Sattler, *et al* 1999). BCR/ABL also activated Grb2 which later associates with Gab2 and Sch following the binding of Grb2 to BCR/ABL protein at an auto-phosphorylation site Y-177 on BCR/ABL (Sattler, *et al* 2002, Sattler, *et al* 1999). The Gab2 is responsible for the activation of the PI3K/AKT and Ras/Raf/MEK/ERK pathways. The Gab2 is also activated by the β_C receptor after engagement, which is evidence of cross-talk between the BCR/ABL and GM-CSF receptor. Therefore, although tyrosine kinase activity of BCR/ABL effectively is blocked by imatinib, CML cells may survive this specific therapy in the presence of cytokines such as GM-CSF.

Many studies have discovered that primitive CML cells isolated from patients can survive and proliferate *in vitro* in the absence of growth factors. We also have shown previously in our experiments that CML but not normal $CD34^+$ cells have spontaneous proliferation in cultures without added serum. Previously, it was suggested that the autonomous growth is consistently associated with, and at least partly dependent on, an abnormal activation of IL-3 and G-CSF as an autocrine mechanism (Jiang, *et al* 2000, Jiang, *et al* 1999). The data on such autocrine models are still conflicting because Zhang and Ren (1998) and Li et al (1999) have shown that a proportion of mice with CML-like disease, induced by BCR/ABL, have detectable levels of IL-3 and/or GM-CSF in their serum by using improved techniques to induce CML in mouse models (Li, *et al* 1999, Zhang and Ren 1998). Some observations also suggested that the growth factor independence has been associated with autocrine production of IL-3 and GM-CSF (Daley and Baltimore 1988, Kabarowski, *et al* 1994, Lugo and Witte 1989), whereas the findings on primary CML cells from patients in chronic phase showed that the growth characteristics of CML progenitors were very similar to normal progenitors, with normal response to growth factor and no evidence of growth factor independence (Eaves, *et al* 1986). However, when examining Ph^+ and Ph^- compartments separately, Ph^+ cells are actively cycling whether cultured in the presence or absence of marrow stroma (Eaves, *et al* 1986).

Autocrine/paracrine cytokines may be a key mechanism allowing CML stem cell survival during imatinib therapy. Previous *in vitro* studies have shown imatinib was unable to kill all CML progenitors, and some of the non-divided cells still survived although no further proliferation was observed (Holtz, *et al* 2002). Thus autocrine cytokines may maintain cell survival in the non-divided stage.

4.2. *Aims of the study*

This experiment aims to examine;

4.2.1. The ability of the IL-3 superfamily cytokine GM-CSF to protect CML cells from the BCR/ABL-specific blocking agent imatinib.

4.2.2. The role of BCR/ABL compared with autocrine GM-CSF on the spontaneous growth of CML CD34+ cells

4.2.3. The ability of the specific GM-CSFα receptor blocking agent E21R to reduce the *in vitro* GM-CSF stimulation of cell proliferation and viability.

4.3. *Special method*

4.3.1. Colorimetric MTS (3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyl tetrazolium bromide) assay

Cryopreserved CML samples from 17 patients in chronic phase were thawed and centrifuged over a Lymphoprep gradient to remove dead cells and obtain the mononuclear cell fraction (MNC). Using a magnetic separation kit (MACS CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec, Ca, USA.), CD34+cells were selected with a purity of ≥ 95% confirmed by flow cytometry. The selected CML CD34+cells were cultured in 96-well plates at a density of $4x10^4$ cells/well using 4 different culture conditions containing SDM, SDM+10μg/ml E21R, SDM+300pg/ml GM-CSF and SDM+300pg/ml GM-CSF+10μg/ml E21R. Imatinib was titrated over the range 0-10 μM in each condition as indicated above. Plates were incubated for 3 days at 37^0C in a humidified atmosphere with 5% CO_2. Wells were analysed using MTS assay (Cell Titer 96 Aqueous One Solution Cell

Proliferation Assay, Promega, USA.) which is a simple colorimetric method of determining relative number of viable cells(Mosmann 1983). 20μL reagent was added per 100μL culture medium, and cells were incubated for 4 hours before analysis. Metabolically active cells bioreduce the tetrazolium compound to a coloured formazan product, which is read by the ELISA plate reader at 490nm, with absorbance correlating to the number of viable cells.

4.4. *Statistical method and patient characteristics*

All CML cells used in this experiment were $CD34^+$ cells derived from 21 patients with chronic phase including 8 females and 13 males. The median age of patients was 47.6 ± 13.0 years old. Basic data contained Haemoglobin level = 9.2 ± 3.0 g/dL, White cell count = 239.7 ± 188.6 x 10^9 / L, percent myeloblasts in marrow = 5.8 ± 3.8 %, percent FISH for BCR/ABL positive cells = 91.0 ± 6.2 % and the IC50 of decrease of CRKL phosphorylation by imatinib = 0.47 ± 0.20 μM. All the values were derived from Mean ± SD.

The statistical method as shown in the results was presented as Mean ± 95%CI or mean with SD where significance was determined as no overlapping within the intervals of the compared-values. The overall analysis of viability and proliferation index used two-way ANOVA to compare the means of proliferation index and percent viability of CML $CD34^+$ cells in the presence of independent treatment variables including E21R, GM-CSF and imatinib.

4.5. *Results*

4.5.1. *A time-course comparison of CML proliferation in response to GM-CSF*

To consider the time-point for the cross-sectional studies on the effect of GM-CSF and imatinib on CML $CD34^+$ cells in terms of cell proliferation and viability, the primary CML cells were incubated after CFSE stain overnight as described in Chapter 3. Then labeled cells were sorted using PE-conjugated CD34 and 7-AAD to obtain the live CML $CD34^+$ cells which were CD34-PE^+/ 7-AAD^-/ $CFSE^+$. 5x10^5 sorted cells were seeded in 24 well-

plates in SDM supplemented with or without 300 pg/mL GM-CSF. The labeled cells were cultured in 37 °C humidified-incubators at 5% pCO_2 over 6 days. Cells were harvested every second day to evaluate cell proliferation, viability and absolute cell number as shown in Figures 4.1, panel a. to c.

The cell viability by 7-AAD exclusion provided some interesting features of the growth of these cells, as shown in Figure 4.1, panel a. In the first 4 days, GM-CSF gave an enhancement in cell viability, when the cells with or without GM-CSF were compared. However, after 4 days, the non-treated cells had cell viability similar to the GM-CSF-treated cells. Therefore, to evaluate cell viability after 4 days of culture because of the overall low viability of cells, may mask the enhancing effect of GM-CSF on BCR/ABL driven proliferation. The masking effect might result in obtaining similar viability values between the non-treated cells and the GM-CSF-treated cells.

The result of the proliferation study on CML $CD34^+$cells, as shown in Figure 4.1, panel c, demonstrated that GM-CSF induced cell proliferation augmenting the spontaneous proliferation due to BCR/ABL after 2 days culture. These cells had an increased proliferation index over the non-treated cells. After 2 days, the proliferation features of cells in two conditions of treatment could be distinguished.

The absolute number of live cells, which are arisen from the balance between cell proliferation and apoptosis, as shown in Figure 4.1, panel b suggested that cells with SDM had sufficient cell death over 4 days to exceed the ability of spontaneously occurring proliferation to maintain viable cell number.

This is in contrast to the cells treated with GM-CSF. The live cell number in culture with GM-CSF was increased over the 4 days of culture. The decline in cell viability in culture with GM-CSF (in panel a, Figure 4.1) after 4 days is likely to be from cell overgrowth. In subsequent experiments, 3 days was chosen as a standard culture condition to avoid artefacts due to well overgrowth.

4.5.2. *The spontaneous growth of CML $CD34^+$ cells is effectively blocked by imatinib. However, GM-CSF could reverse this situation.*

Figure 4.1. The time-course experiment with CFSE to evaluate the cell proliferation, viability and absolute number of live cells.

CML $CD34^+$ from a patient in chronic phase were sorted using CFSE, 7-AAD and CD34-PE. Sorted cells were seeded in SDM only (□), and SDM with 300 pg/mL GM-CSF(X) over 6 days. Cells were then harvested every other day to evaluate cell viability, absolute cell number and cell proliferation.

The panel a showed that CML $CD34^+$ cells in both SDM and SDM with GM-CSF had overall decrease of cell viability while the absolute number of cells in panel b and cell proliferation in panel c was increased. The absolute number of cells represent the balance between total cell proliferation and cell viability. The decrease of cell viability after 4 days in SDM with GM-CSF was slower than cells in SDM only was also observed.

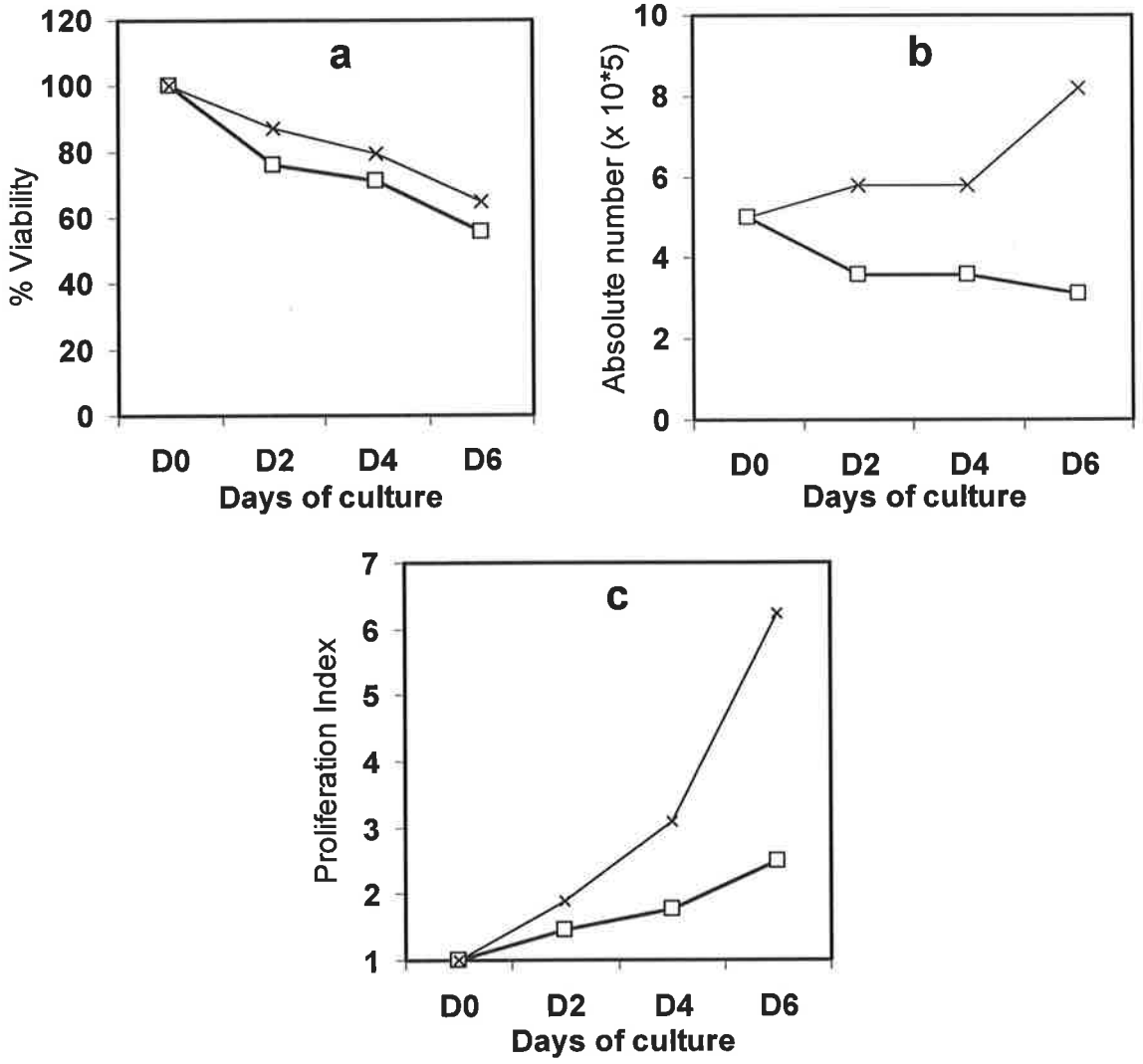

a
% Viability
120
100
80
60
40
20
0
D0
D2
D4
D6
Days of culture
b
Absolute number (x 10*5)
10
8
6
4
2
0
D0
D2
D4
D6
Days of culture
c
Proliferation Index
7
6
5
4
3
2
1
D0
D2
D4
D6
Days of culture

Figure 4.1

CML CD34$^+$ cells showed spontaneous proliferation in the absence of added cytokines as shown in Figure 4.2, panel a., where significant events were seen in the peaks representing one and two division cycles after 3 days of cultures. The movement of cells into the peaks representing divided cells was reduced by 1μM imatinib (Figure 4.2, panel b.), suggesting that the spontaneous proliferation was due to the activity of the BCR/ABL protein. When GM-CSF was added to the CML CD34$^+$ cell culture, it increased the size of peaks at divisions 1-4 and decreased the size of the parental peak at division 0 (Figure 4.2, panel c.) compared with CML CD34$^+$ cells without treatment (Figure 4.2, panel a.), suggesting that GM-CSF functioned additively with active BCR/ABL in the stimulation of proliferation. Interestingly, when GM-CSF with imatinib were added to cultures (Figure 4.2, panel d.), the proliferation peaks were similar to those exhibited by CML CD34$^+$ cells without treatment (Figure 4.2, panel a.). The spontaneous activity of CML CD34$^+$ cells was unlikely to be due to a local autocrine/paracrine effect because it was completely blocked by imatinib. The recovery of proliferation after adding GM-CSF suggests that the replacement of signal activation by GM-CSF receptor occurred when BCR/ABL tyrosine kinase activity was blocked by imatinib.

4.5.3. *GM-CSF prevents imatinib suppression of proliferation of CML CD34$^+$ cells.*

In Figure 4.3, panel a, imatinib was shown to effectively reduce the spontaneous growth or proliferation of CML CD34$^+$ cells, particularly at concentrations ≥ 1μM. The CML CD34$^+$ cells which were treated with imatinib only, at concentrations titrated from 0 to 10 μM, were used as controls. In the presence of 300 pg/mL GM-CSF in cultures as previously described in Chapter 3, the proliferation index of CML CD34+ cells (PI= 1.25±0.021, n=5, 95% CI) could be recovered to a level significantly higher than the imatinib only treated cells even in the cultures with high concentration of imatinib (10 μM) (1.105±0.039, n=5, 95% CI). Furthermore, the level of PI of cells in culture with GM-CSF at the point of 10 μM imatinib (PI= 1.25±0.021, n=5, 95% CI) is lower than the cells in culture without any added treatment (they should be exactly in the same level if GM-CSF fully replaced the activity of BCR/ABL), but no statistical difference was found (1.363±0.158, n=5, 95% CI). This is because GM-CSF may only partially replace the function of BCR/ABL activity involving the cell proliferation.

Figure 4.2. GM-CFS could reverse the effect of imatinib on inhibition of proliferation activity of CML CD34+ cells

CML CD34+ cells exhibit spontaneous proliferation (panel a), which is driven by the BCR/ABL protein. When imatinib is added to the culture, the proliferation activity is clearly decreased as shown in panel b compared with panel a. GM-CSF is not needed for CML CD34+ proliferation but it provides additional signalling additive to BCR/ABL protein (panel c compared with panel a). We can see that the proliferation activity is increased to a similar level as the spontaneous proliferation in panel a. after adding GM-CSF to the culture with imatinib (panel d compared with a).

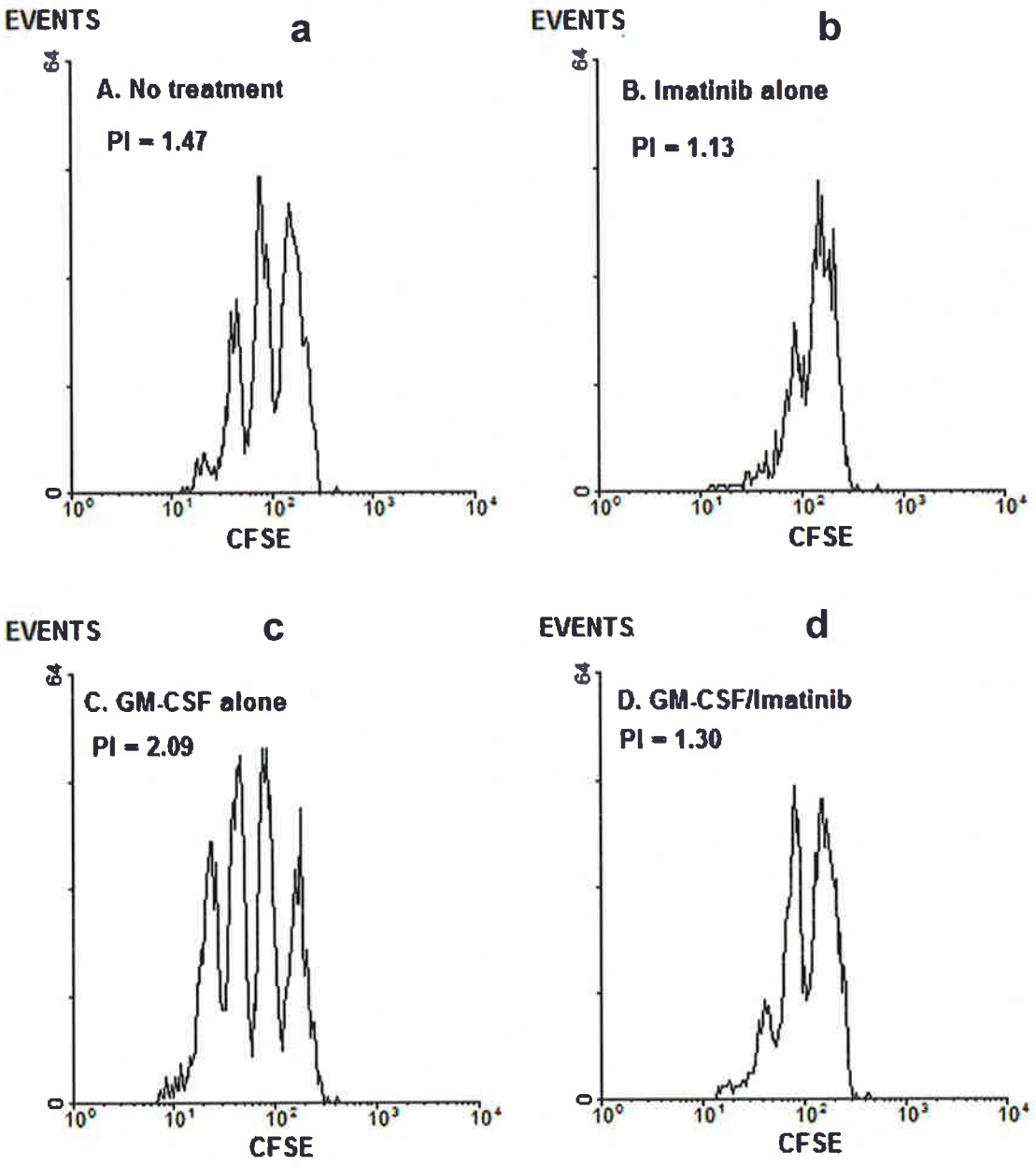

EVENTS
a
64
A. No treatment
PI = 1.47
0
10⁰ 10¹ 10² 10³ 10⁴
CFSE
EVENTS
b
64
B. Imatinib alone
PI = 1.13
0
10⁰ 10¹ 10² 10³ 10⁴
CFSE
EVENTS
c
64
C. GM-CSF alone
PI = 2.09
0
10⁰ 10¹ 10² 10³ 10⁴
CFSE
EVENTS
d
64
D. GM-CSF/Imatinib
PI = 1.30
0
10⁰ 10¹ 10² 10³ 10⁴
CFSE

Figure 4.2

To demonstrate it is a GM-CSF specific effect overriding imatinib, the specific GM-CSFα receptor antagonist E21R was used to block the activation of GM-CSF receptor by GM-CSF. E21R was demonstrated to bring the proliferation index of the cells in cultures with GM-CSF down to the same level as the control at each individual point of imatinib. This suggests E21R completely blocks the binding of GM-CSF to its receptors and GM-CSF could provide the signal stimulation which had already been inhibited by imatinib. The data have been shown in Table 4.1.1.

Two-way unrelated ANOVA showed that imatinib was effective in reduced cell proliferation within the combined treatment groups ($F_{5,96}$ = 13.690, $p < 0.001$), and significant effects were also obtained among the combined treatment groups ($F_{3,96}$ = 52.007, $p < 0.001$) which was mentioned in the cells treated with imatinib and GM-CSF found having significantly higher PI than other groups (Table 4.1.2).

4.5.4. *GM-CSF could improve cell viability in the presence of imatinib.*

At imatinib 0 μM in Figure 4.3, panel b, although GM-CSF could not improve cell viability over the control (viablility of control = 38.35±14.9%, n=5, with 95% CI), the cells with GM-CSF only had the highest viability (64.0±13.0, n=5 with 95% CI) which was suppressed to the same level as the control, when E21R was included (43.0±14.5, n=5, with 95% CI). This is similar to what was found when examining the time-course viability, which showed that no significant difference occurred during day 2-4 between the GM-CSF treated cells and non-GM-CSF cells (Figure 4.1, panel a) but in contrast there was a large difference in proliferation index (Figure 4.1, panel c). The viability of cells in the cultures with GM-CSF at every point of controls (cultures with imatinib alone), which were the cells treated with imatinib only, was also higher than the control and significantly higher than the control when doses of imatinib ≥ 0.1 μM. The GM-CSF effect was also reversed to the control level with added E21R suggesting that the improved cell viability was the effect of GM-CSF. The results on viability of CML CD34$^+$ cells are compatible with the findings on proliferation index in Figure 4.3, panel a.

Figure 4.3. GM-CSF restores CML CD34$^+$ cell proliferation and maitains cell viability in the presence of imatinib

CML CD34$^+$ cells derived from 5 patients in chronic phase were seeded in SDM for 3 days with imatinib over 0 - 10 μM with or without 300 pg/mL GM-CSF with or without 10 mg/mL E21R. The mean proliferation (panel a) and percent viability (panel b) of CML CD34$^+$ cells were effectively suppressed by imatinib (red bar).

Panel a, GM-CSF enhances PI at all concentrations of imatinib, such that even at the highest levels, PI was not significantly different to basal or spontaneous proliferation. The proliferative enhancement by GM-CSF was completely blocked by E21R.

Panel b, percent viability of cells which is identified by the percentage of 7-AAD negative cells within the total CFSE+/ CD34-PE+ cells has been calculated. Although GM-CSF could maintain the viability of cells (white bar) at higher level than non-treated cells (dash line), there is no statistical difference between GM-CSF treated group and non-treated group. The basal cell viability which was defined by the viability of non-treated cells was maintained by GM-CSF in the presence of imatinib even at 10 μM imatinib.

The dash lines shown in both panels represent the level of PI and percent viability of non-treated control.

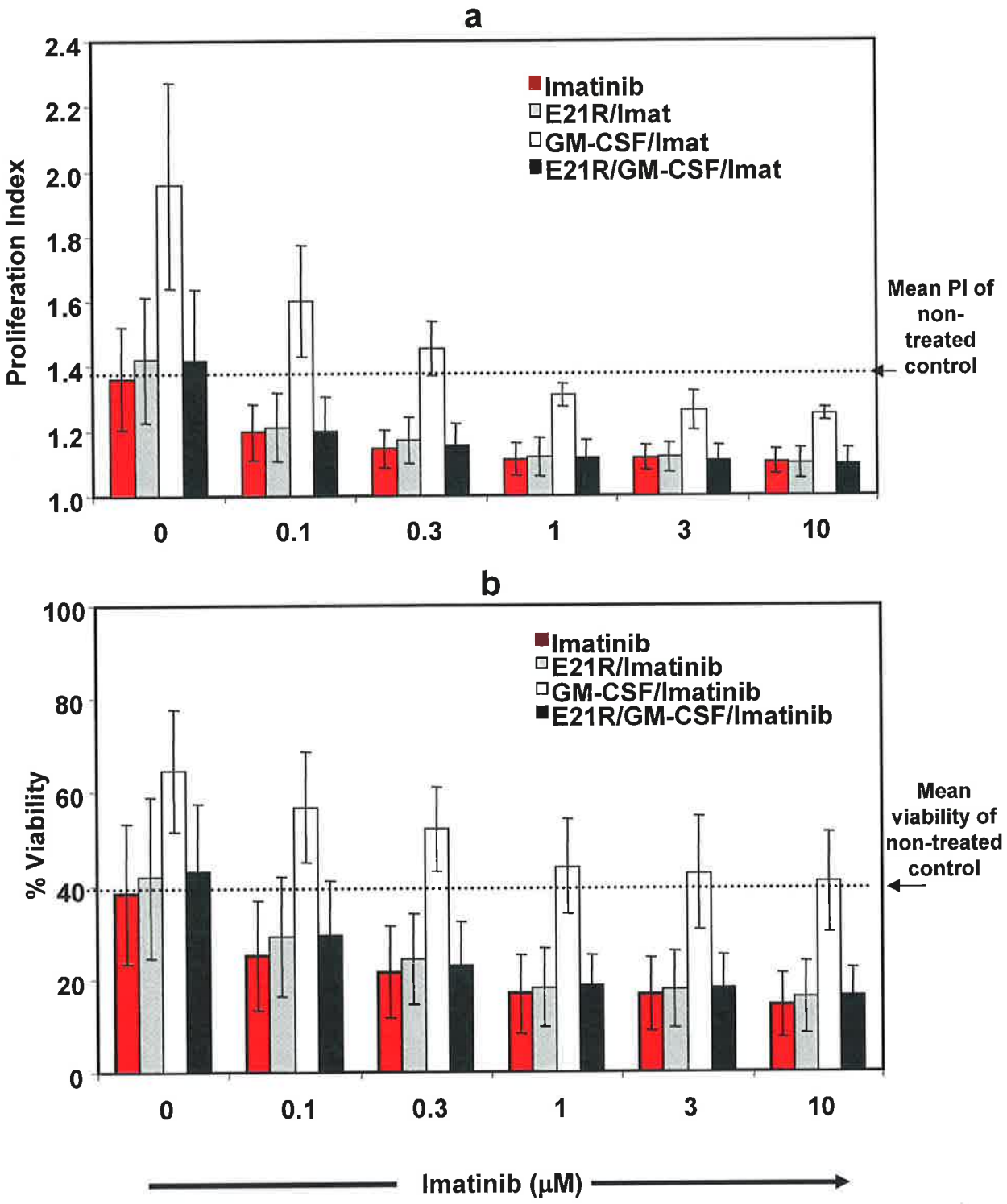

a
Proliferation Index
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Imatinib
E21R/Imat
GM-CSF/Imat
E21R/GM-CSF/Imat
Mean PI of non-treated control
0
0.1
0.3
1
3
10
b
% Viability
100
80
60
40
20
0
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imatinib
Mean viability of non-treated control
0
0.1
0.3
1
3
10
Imatinib (μM)

Figure 4.3

Table 4.1.1 The proliferation index of CML CD34$^+$ cells shown in Figure 4.3, panel a

Proliferation Index								
treatment	A	B	C	D	E	Mean	SD	95% CI
EGI 0	1.212	1.560	1.200	1.497	1.115	1.419	0.192	0.218
EGI 0.1	1.140	1.266	1.087	1.241	1.059	1.198	0.097	0.110
EGI 0.3	1.128	1.191	1.086	1.187	1.043	1.155	0.059	0.067
EGI 1	1.133	1.137	1.060	1.148	1.034	1.115	0.048	0.054
EGI 3	1.111	1.137	1.060	1.125	1.035	1.107	0.042	0.047
EGI 10	1.100	1.126	1.045	1.117	1.026	1.096	0.045	0.051
GI 0	1.501	2.105	1.635	2.132	2.058	1.957	0.280	0.316
GI 0.1	1.351	1.688	1.426	1.690	1.913	1.602	0.152	0.172
GI 0.3	1.279	1.476	1.370	1.515	1.816	1.454	0.075	0.085
GI 1	1.219	1.305	1.282	1.345	1.657	1.311	0.032	0.036
GI 3	1.246	1.261	1.210	1.317	1.666	1.263	0.054	0.061
GI 10	1.282	1.239	1.239	1.271	1.570	1.250	0.019	0.021
EI 0	1.205	1.550	1.228	1.481	1.061	1.420	0.169	0.192
EI 0.1	1.144	1.280	1.108	1.250	1.036	1.213	0.092	0.104
EI 0.3	1.143	1.217	1.101	1.194	1.022	1.171	0.062	0.070
EI 1	1.120	1.157	1.061	1.141	1.014	1.119	0.051	0.058
EI 3	1.116	1.147	1.072	1.133	1.018	1.117	0.040	0.045
EI 10	1.132	1.131	1.052	1.115	1.015	1.100	0.042	0.047
I 0	1.179	1.467	1.204	1.418	1.045	1.363	0.140	0.158
I 0.1	1.148	1.262	1.113	1.218	1.024	1.198	0.077	0.087
I 0.3	1.151	1.186	1.089	1.164	1.022	1.146	0.051	0.057
I 1	1.135	1.146	1.064	1.130	1.014	1.113	0.043	0.049
I 3	1.147	1.140	1.078	1.131	1.008	1.116	0.033	0.038
I 10	1.150	1.130	1.066	1.119	1.016	1.105	0.034	0.039

I = Imatinib , E = 10 μg/mL E21R, G = 300 pg/mL GM-CSF
0, 0.1, 0.3, 1, 3, 10 are the concentrations of imatinib in μM.
A, B, C, D and E represented 5 CML patients in chronic phase.

Table 4.1.2. Two-way ANOVA analysis of proliferation index

For Table 4.1.1 and Figure 4.3 a

Tests of Between-Subjects Effects

Dependent Variable: Prolif

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.540(a)	23	.197	10.687	.000
Intercept	182.669	1	182.669	9889.970	.000
treatment	2.882	3	.961	52.007	.000
Imatinib	1.264	5	.253	13.690	.000
treatment * Imatinib	.394	15	.026	1.423	.152
Error	1.773	96	.018		
Total	188.982	120			
Corrected Total	6.313	119			

a R Squared = .719 (Adjusted R Squared = .652)

"treatment" means the combination of therapy including:

- E21R/GM-CSF/Imatinib
- GM-CSF/Imatinib
- E21R/Imatinib
- Imatinib

Table 4.2.1. Viability of CML CD34$^+$ cells shown in Figure 4.3, panel b

Percent Viability								
treatment	A	B	C	D	E	Mean	SD	95%CI
EGI 0	33.599	52.718	35.704	66.920	26.110	43.010	16.534	14.493
EGI 0.1	24.870	33.932	20.857	50.440	16.960	29.412	13.337	11.690
EGI 0.3	21.282	23.245	18.637	40.060	10.290	22.703	10.888	9.544
EGI 1	15.404	17.478	15.649	31.290	12.920	18.548	7.305	6.403
EGI 3	15.047	17.039	17.258	31.260	9.470	18.015	8.043	7.050
EGI 10	13.863	14.168	16.327	27.520	9.800	16.336	6.682	5.857
GI 0	59.462	71.045	44.058	84.380	64.500	64.689	14.842	13.009
GI 0.1	48.938	55.051	39.834	73.620	66.610	56.810	13.527	11.856
GI 0.3	46.732	43.767	43.353	64.620	61.600	52.014	10.268	9.000
GI 1	37.394	31.908	38.254	53.230	58.910	43.939	11.515	10.093
GI 3	38.830	28.418	31.718	53.720	60.060	42.549	13.804	12.100
GI 10	37.110	24.805	35.394	53.030	52.770	40.622	12.159	10.658
EI 0	32.127	54.977	29.406	69.580	22.640	41.746	19.746	17.308
EI 0.1	25.641	33.373	19.267	51.840	14.910	29.006	14.537	12.742
EI 0.3	20.302	26.436	19.312	42.180	12.790	24.204	11.153	9.776
EI 1	14.963	18.065	13.583	34.250	9.250	18.022	9.609	8.423
EI 3	14.815	16.737	13.765	33.810	9.260	17.677	9.427	8.263
EI 10	12.848	13.480	13.044	31.180	8.800	15.870	8.764	7.682
I 0	27.703	49.526	27.378	63.000	24.150	38.351	17.087	14.978
I 0.1	19.396	30.298	16.432	46.080	13.610	25.163	13.293	11.652
I 0.3	17.600	23.058	16.972	39.670	9.840	21.428	11.227	9.841
I 1	14.096	17.086	13.224	32.210	6.750	16.673	9.468	8.299
I 3	12.815	16.300	13.125	31.810	8.720	16.554	8.943	7.839
I 10	10.873	12.665	12.322	27.750	7.960	14.314	7.737	6.781

I = Imatinib , E = 10 μg/mL E21R, G = 300 pg/mL GM-CSF
0, 0.1, 0.3, 1, 3 and 10 are the concentrations of imatinib in μM.
A, B, C, D and E represented 5 CML patients in chronic phase.

Table. 4.2.2. Two-way ANOVA analysis of % viability for

Table 4.2.1 and Figure 4.3 b

Tests of Between-Subjects Effects

Dependent Variable: viability

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24970.356(a)	23	1085.668	7.405	.000
Intercept	110346.610	1	110346.610	752.668	.000
treatment	15791.667	3	5263.889	35.905	.000
Imatinib	9046.508	5	1809.302	12.341	.000
treatment * Imatinib	132.181	15	8.812	.060	1.000
Error	14074.304	96	146.607		
Total	149391.270	120			
Corrected Total	39044.660	119			

a R Squared = .640 (Adjusted R Squared = .553)

"treatment" means overall analysis of all treatments including :

- E21R/GM-CSF/Imatinib
- GM-CSF/Imatinib
- E21R/Imatinib
- Imatinib

Two-way unrelated ANOVA showed that imatinib was effective in reduced cell viability within the combined treatment groups ($F_{5,96}$ = 12.341, p<0.001), and significant effects were also obtained among the combined treatment groups ($F_{3,96}$ = 35.905, p < 0.001) which was mentioned in the cells treated with imatinib and GM-CSF found having significantly higher percent viability than other groups (Table 4.2.2) suggesting some combined treatment groups, which should be the cells treated with GM-CSF and imatinib, had significant difference of percent viability from others.

4.5.5. *Analysis of the protective effect of GM-CSF on CML CD34$^+$ cell cultures treated with imatinib using the MTS assay.*

CML CD34$^+$ cells from 17 patients were used to examine proliferation activity changes monitored by MTS reduction under different culture conditions. The MTS assay was chosen as a high throughput screening process, to determine if the measurement of total viable cells in cultures gave comparable results to the more labour intensive dye dilution assays of CML CD34$^+$ cells cultured in SDM-based medium. Adding GM-CSF (mean = 0.77, SD = 0.26, n=17) resulted in a significantly higher absorbance than for CML CD34$^+$ cells treated with imatinib only (mean = 0.5, SD= 0.2, n=17) as shown in Table 4.3.1. In addition, E21R brought the curve of absorbance (mean = 0.51, SD = 0.2, n=17) down to the same level as cells treated with imatinib. The MTS results on selected CML CD34$^+$ cells were in broad agreement with the CFSE analysis results (Figure 4.4).

The overall assessment by two-way ANOVA showed significant effects were obtained for imatinib treatment within each combined-treatment group ($F_{5,384}$ = 36.482, p < 0.001) and among the treatment groups ($F_{3,384}$ = 36.881, p < 0.001) as shown in Table 4.3.2.

4.5.6. *Consideration of the percent recovery or viability of parent cells*

Parent cells number is calculated from the conversion of the absolute number of cells at the end of the culture period to the number of original input cells. The live and total CFSE-labeled cells in cultures will be analysed separately using 7-AAD stain and high-resolution fluorescence beads in order to calculate the absolute number and percent viability of parent cells. If there is no cells loss, the total derived parent cell number should be equal to the

Figure 4.4. MTS assay to evaluate the proliferation of bulk CML CD34$^+$ cells confirmed GM-CSF protection against imatinib.

Cells from 17 patients in chronic phase were cultured to examine the effect of GM-CSF on imatinib suppression of primary CML CD34+ cells. GM-CSF enhanced the survival of CML CD-34+ cells at all doses of imatinib. The dashed line is the mean level of the control cells Error bars are mean ± 95% CI. The corrected OD is blank subtracted OD. The blank meant that the well contained only SDM without cells.

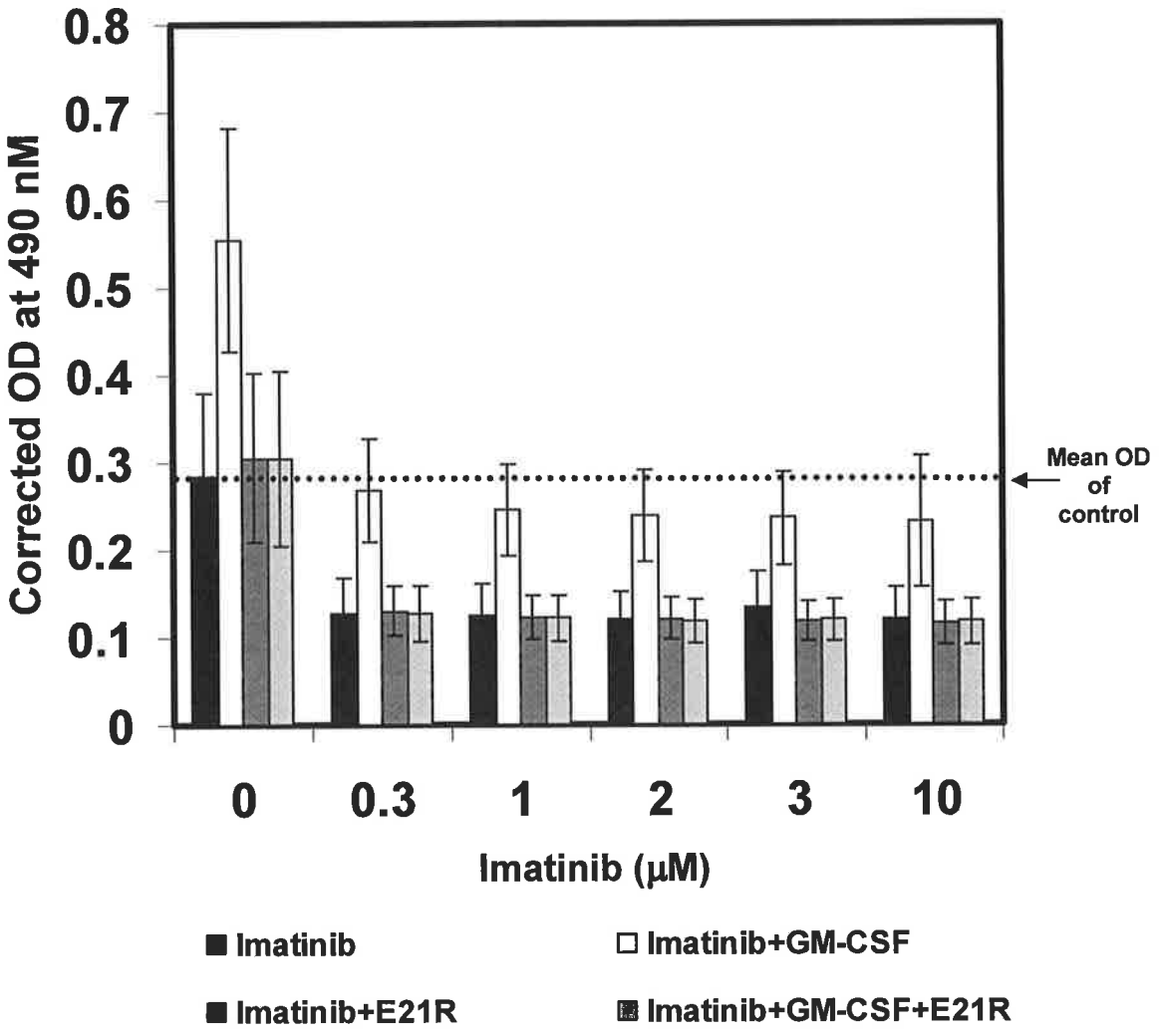

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Corrected OD at 490 nM
Mean OD of control
0
0.3
1
2
3
10
Imatinib (μM)
Imatinib
Imatinib+GM-CSF
Imatinib+E21R
Imatinib+GM-CSF+E21R

Figure 4.4

Table 4.3.1. Mean OD on MTT assay

Descriptive Statistics

Dependent Variable: OD

Treatment	imatinib	Mean	Std. Deviation	N
EGI	0	.51288	.196707	17
	0.3	.34500	.056583	17
	1	.34035	.049365	17
	2	.33776	.046455	17
	3	.33888	.044227	17
	10	.33335	.040446	17
	Total	.36804	.110378	102
GI	0	.77435	.266096	17
	0.3	.48800	.125386	17
	1	.46482	.110229	17
	2	.45841	.109084	17
	3	.45541	.110544	17
	10	.40971	.158299	17
	Total	.50845	.195577	102
EI	0	.51329	.203289	17
	0.3	.34800	.062553	17
	1	.34106	.053814	17
	2	.34006	.049197	17
	3	.33776	.047507	17
	10	.33182	.043280	17
	Total	.36867	.113626	102
I	0	.50353	.199586	17
	0.3	.34747	.084553	17
	1	.34465	.076126	17
	2	.34112	.066562	17
	3	.35424	.085611	17
	10	.33776	.062276	17
	Total	.37146	.119950	102
Total	0	.57601	.242601	68
	0.3	.38212	.104665	68
	1	.37272	.091824	68
	2	.36934	.087628	68
	3	.37157	.089975	68
	10	.35316	.093994	68
	Total	.40415	.151415	408

Table 4.3.2. Two-way ANOVA analysis of MTT assay

Tests of Between-Subjects Effects

Dependent Variable: OD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.194(a)	23	.182	13.633	.000
Intercept	66.643	1	66.643	4982.006	.000
Treatment	1.480	3	.493	36.881	.000
imatinib	2.440	5	.488	36.482	.000
Treatment * imatinib	.274	15	.018	1.367	.160
Error	5.137	384	.013		
Total	75.974	408			
Corrected Total	9.331	407			

a R Squared = .450 (Adjusted R Squared = .417)

"treatment" means the combination therapy including:

- E21R/GM-CSF/Imatinib
- GM-CSF/Imatinib
- E21R/Imatinib
- Imatinib.

total input cells at the initiation of cultures (Day 0). The decrease of percent viability of parent cells will be due to the treatment condition as well as environmental factors which required the non-treatment culture control to assess the spontaneous cell death. By using the same concept as the colony culture assay i.e. one active progenitor will create one colony, the IC_{50} of the different culture conditions can be calculated.

We found three different types of responses to the combination of imatinib and GM-CSF when the proliferation activity of CML CD34+ cells was examined, and the data on percent recovery or viability of parent cells was compared (Figures 4.5 to 4.7).

Patient 1, Figure 4.5 (Panel a.), the number of live parent cells in control cultures decreased spontaneously during culture to around 25%. The added GM-CSF raise the percent survival to around 40%, a difference of about 15 percentage points. When examining parent cell viability at 3 μM imatinib, the difference between imatinib plus GM-CSF and imatinib only was around 25 percentage points. Compared with the non-GM-CSF culture, after 0.1 μM imatinib, the viability of parent cells drop about 40% (25% increased points after 3 μM minus 15% increased points at no imatinib, then the difference divided by 25%). This data suggested GM-CSF did not give resistance of cells to imatinib. However, when the percent viability data were normalized (Figure 4.5, Panel b), the cells treated with GM-CSF plus imatinib markedly shifted the imatinib IC_{50} from 0.1 to 1 μM, a 10 fold-difference. This suggested GM-CSF induced cells of this patient to be relatively insensitive to imatinib. This was confirmed by adding GM-CSF + E21R, which shifted the IC_{50} to the basal IC_{50} of the primary CML cells.

Patient 2, Figure 4.6 (Panel a.), the parent cells of this patient had a basal recovery during culture of about 30%. When GM-CSF was added to cultures, it raised the percent recovery to 40%, a difference of about 10 percentage points. When examining at an individual point of imatinib, the difference between cells treated with GM-CSF plus imatinb and imatinib only appears similar. This suggested GM-CSF could allow parent cells to survive imatinib but did not change the sensitivity of cell response to imatinib. This is confirmed by the normalized data which showed a similar IC_{50} (Figure 4.6, Panel b).

Figure 4.5. Evaluation of the percent viability of parent cells of CML $CD34^+$ cells of patient 1

Using dye-dilution of CFSE, the total absolute number of parent cells could be calculated from the total number of cells. 7-AAD distinguished live cells from dead cells, allowing evaluation of the viability of live parent cells after 3 day-cultures, which gave rise to the number of cells in different divisions.

The data from the crude percent viability of parent cells shown in Panel a. demonstrated that first, imatinib could kill the parent cells leaving only 10-15% alive when comparing between non-imatinib and imatinib-treated cells, and second, GM-CSF could maintain the survival of parent cells and keep the number of parent cells approximately at the same level (about 35-40%) over the titrated concentrations of imatinib. These cells after treatment with GM-CSF showed insensitivity to imatinib. These data were confirmed by normalization and found GM-CSF treatment resulted in a marked increase in IC_{50} (Panel b.).

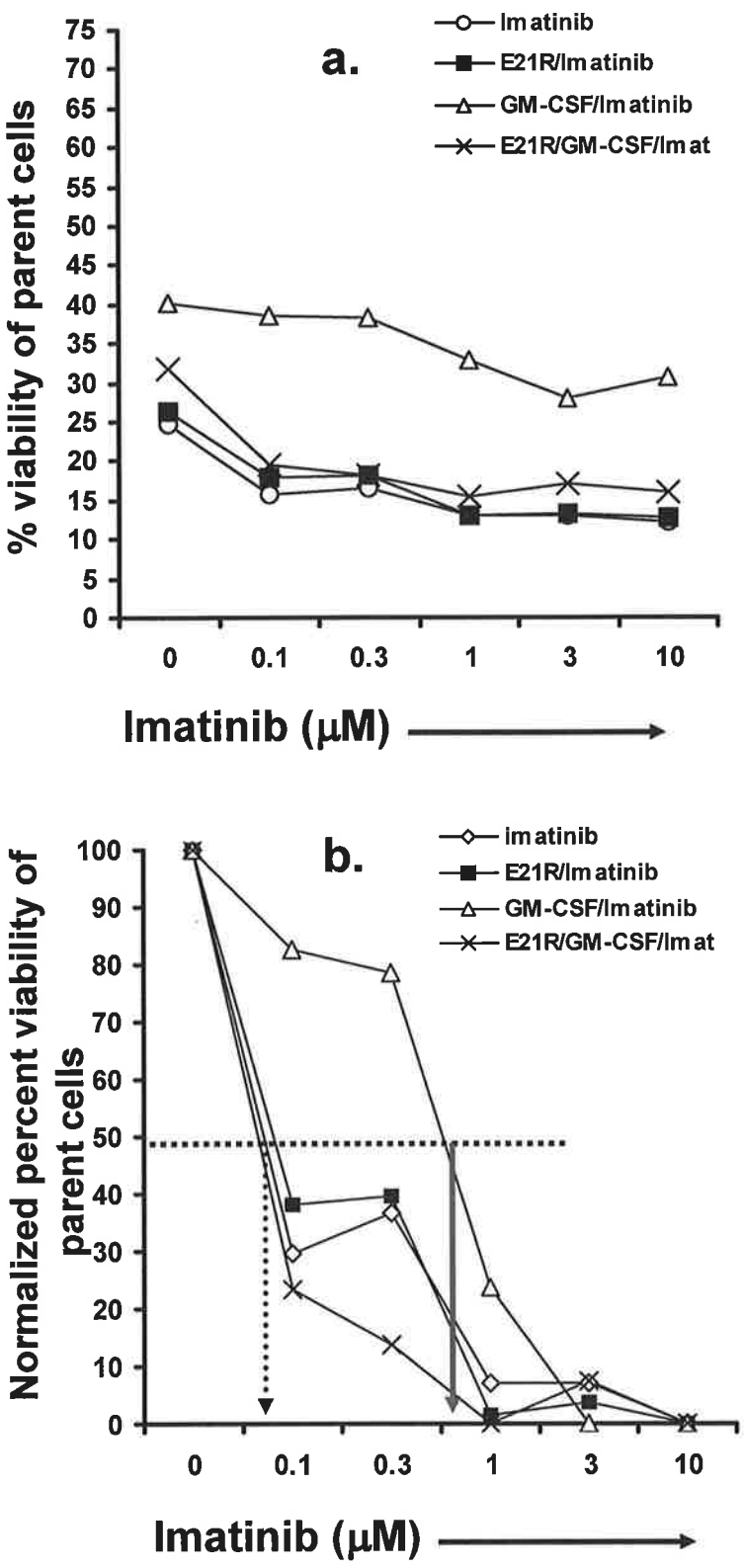

a.
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
% viability of parent cells
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
0.1
0.3
1
3
10
Imatinib (μM)
b.
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
Normalized percent viability of parent cells
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.3
1
3
10
Imatinib (μM)

Figure 4.5

Figure 4.6. The normalized data of CML $CD34^+$ cells derived from patient 2 showed the same pattern of cell response to imatinib in all conditions of treatment.

The viability of parent cells from patient 2 was calculated using the CFSE dye dilution technique panel a and the normalized data on the viability of parent was shown in panel b.

Panel a showed the curve of cells treated with GM-CSF plus imatinib had higher cell viability compared with other conditions, but GM-CSF data showed the curve parallel to other conditions which was different from patient 1 in which the GM-CSF could retain the same viability even at imatinib 10 mM. This suggested that GM-CSF still helped to maintain parent cell survival in the presence of imatinib. These cells were still sensitive to imatinib after adding GM-CSF because of very little increase of IC_{50} (Panel b.).

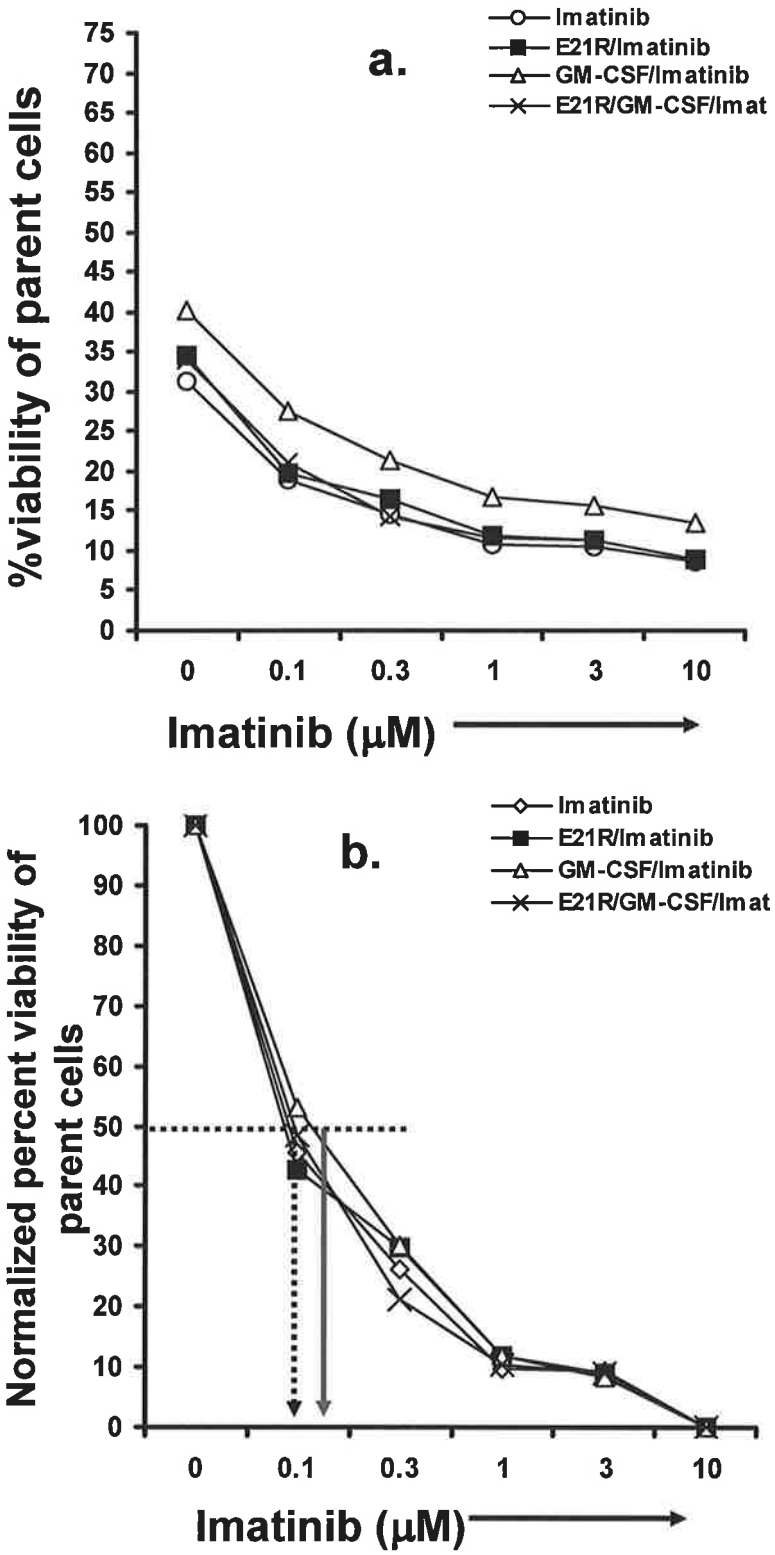

a.
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
%viability of parent cells
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
0.1
0.3
1
3
10
Imatinib (μM)
b.
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
Normalized percent viability of parent cells
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.3
1
3
10
Imatinib (μM)

Figure 4.6

Patient 3, Figure 4.7 (Panel a.), the basal recovery of these cells was high at about 55%, and imatinib at 10 μM could suppress the cell recovery down to around 25% which was still quite high compared with the other two patients. GM-CSF could raise the base line recovery to 70%. This made the difference of percent viability of parent cells between GM-CSF only treated cells and non-treated cells around 15 percentage points. When imatinib was titrated in cultures along with GM-CSF, this combination could increase the difference of percent viability to 20 percentage points from the imatinib only cultures. This finding was in reverse to the result found in patient 1. Therefore the profile suggested that GM-CSF did not induce insensitivity of cells to imatinib. This patient's cells had similar response to imatinib in cultures with or without GM-CSF which was confirmed by normalised data. However, at the concentration of imatinib greater than 1 μM, the viability of parent cells was plateau in the cultures with imatinib plus GM-CSF, while other treatment conditions still marginally lowered the viability. This suggested that these cells had some intrinsic factor to maintain insensitivity to imatinib, because when imatinib greater than 1 μM the viability of parent cells is only marginal decreased. Interestingly, GM-CSF could additively enhance this intrinsic factor.

4.5.7. *The titration of GM-CSF showed that it could replace the signal stimulation blocked by imatinib in CML CD34$^+$ cells.*

Four new chronic-phase CML patients' cells were used to evaluate CML CD34$^+$ cell proliferation using CFSE dye dilution to ensure that the effect of GM-CSF to replace the activity of BCR/ABL was general. Because of the limited stocks of CML cells, the previous patient cells could not be used in this experiment. However, if the effect of GM-CSF on chronic phase CML CD34$^+$ cells was general, the trend of responses of all CML CD34$^+$ should be similar. Sorted CD34-PE$^+$/ 7-AAD$^-$ CFSE labeled CML cells were seeded in 96-well plates at the densitiy of 5 x 10^4 cells/mL. GM-CSF was titrated in cultures over the range 0 – 10,000 pg/mL. The cultures were first added with 1 μM imatinib and the specific GM-CSFRα antagonist-E21R at 10 pg/mL. Then cultures were incubated in the 37 ^{o}C- incubator for 1 hour before adding GM-CSF.

Figure 4.7. The normalization of percent parent cells of CML 3 also showed the same responses to imatinib as in patient 2, Figure 4.6

CFSE labeled CML $CD34^+$ cells from the third patient was cultured in SDM as the same method shown in Figure 4.5. After 3 days, absolute cell number was converted to the number of parent cells. Then the percent viability of parent was calculated as in patient 1 and 2 , as shown in panel a, and normalised data of viability was shown in panel b.

The pattern of response shown in panel a for cells treated with GM-CSF plus imatinib appeared parallel to other conditions similar to patient 2 in Figure 4.6, then reached a plateau when the concentration of imatinib greater than 1 mM, whilst other conditions CML $CD34^+$ cells continued reducing the cell viability. This made a greater percentage points from other conditions and also made the pattern of cell response to the treatment reverse of the data of patient 1

Panel b, the normalised data of all conditions did not show the shift of imatinib IC50

Taken together, this suggests that GM-CSF did not induce total insensivity of cells to imatinib. However, at concentration of imatinib able to suppress the function of BCR/ABL, GM-CSF receptor could maintain the cell activity to replace the function of BCR/ABL.

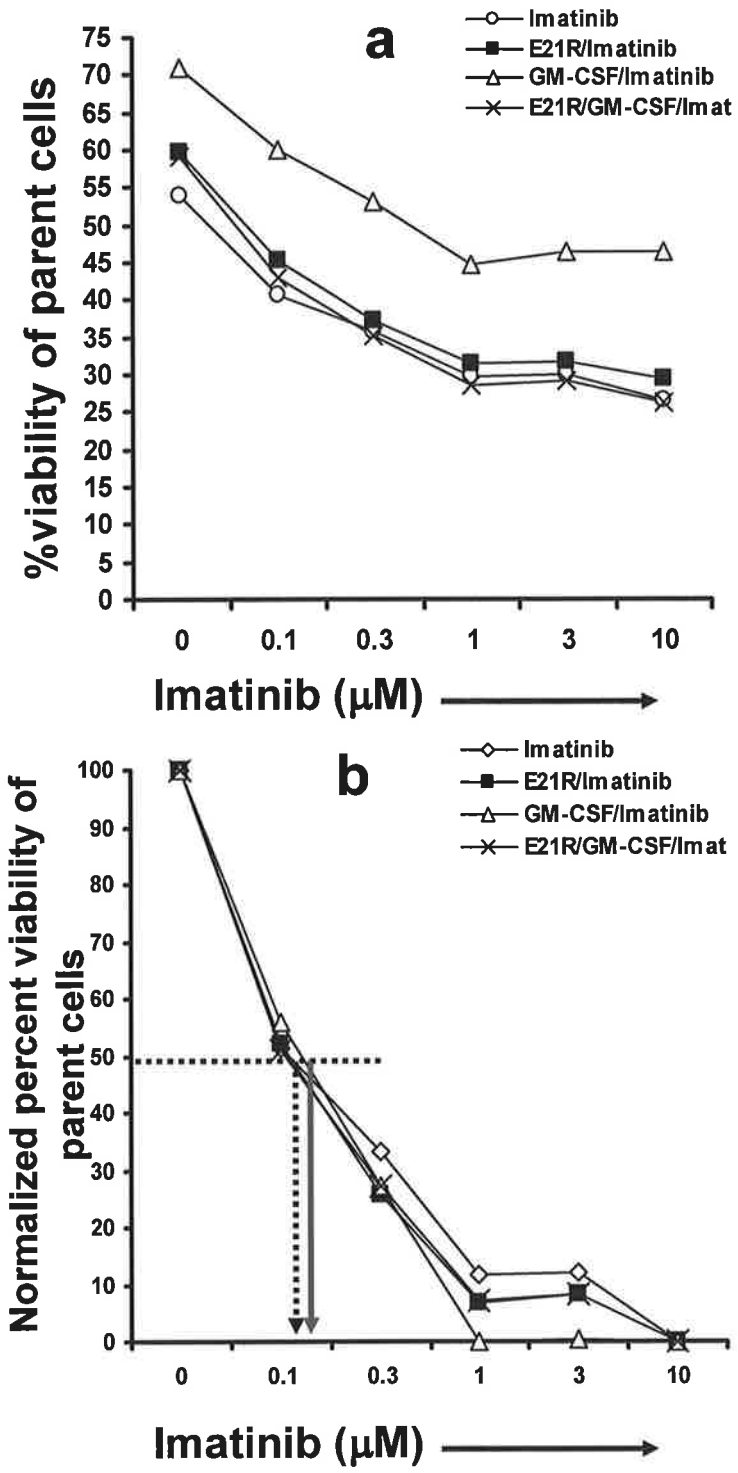

a
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
%viability of parent cells
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
0.1
0.3
1
3
10
Imatinib (μM)
b
Imatinib
E21R/Imatinib
GM-CSF/Imatinib
E21R/GM-CSF/Imat
Normalized percent viability of parent cells
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.3
1
3
10
Imatinib (μM)

Figure 4.7

GM-CSF did not enhance proliferation of CML $CD34^+$ cells at the concentration below 10 pg/mL. However, the cells of imatinib-treated group had a lower PI than those of the non-imatinib-treated group, demonstrating the spontaneous proliferation was driven by BCR/ABL. In both control and imatinib containing cultures, the PI then steadily increased and reached a plateau when GM-CSF was greater than 300 pg/mL. The curves remained parallel as GM-CSF increased, with SDM+GM-CSF+imatinib cultures exhibiting the lower PI due to having only GM-CSF driving their proliferation activity as BCR/ABL was blocked, as shown in Figure 4.8. In contrast, cultures with SDM+GM-CSF and no imatinib had both GM-CSF and BCR/ABL driving their proliferation, and exhibited a higher PI curve. The addition of E21R suppressed the PI enhancement by GM-CSF, with the two parallel curves now showing the same activity as the spontaneous proliferation seen with and without imatinib, showing complete blocking of GM-CSF stimulation.

4.5.8. *Colony assay in semi-solid agar shows GM-CSF protects CML progenitors from imatinib killing as well as their spontaneous death in culture.*

Data shown in table 4.4 and Figure 4.9 indicated that imatinib from 0.1 μM eliminated CML progenitors after 3 days of fluid culture. Evaluation of pre-culture colony forming cells also revealed that there was significant spontaneous loss of colony forming progenitors demonstrating that CML progenitors were not fully growth factor independent. Addition of GM-CSF in the liquid pre-culture enabled significant recovery of progenitors even when 10 μM imatinib was used. Interestingly, although E21R is a very specific antagonist of GM-CSF, it appeared to only partially block the protective effect of GM-CSF on imatinib killing of progenitors. In addition, controls where E21R alone was added to the liquid pre-culture showed that E21R had a slight protective effect, which suggests that binding of E21R to the GM-CSF α chain may be able to transduce survival signals which only required low levels of receptor occupancy by GM-CSF.

4.6. ***Discussion***

Proliferation index was calculated through both live and dead cells which had been described in Chapter 3. Therefore, when the cells showed high proliferative activity, the absolute number and viability of cells could be in contrast to proliferation index. The

Figure 4.8. GM-CSF augments BCR/ABL stimulus to maintain CML CD34+ cell proliferation in the presence of imatinib.

GM-CSF titration was used to examine the contribution of BCR/ABL activity and GM-CSF signalling to the proliferation index of CML CD34+ cells. CD34+ cells from 4 different CML patients were CFSE stained and set up in culture for three days under conditions indicated in the figure and the Proliferative Index calculated after flow cytometric analysis. Imatinib was used at 1 μM and E21 used at 10 μg/ml . Imatinib and E21R inhibition of BCR/ABL and GM-CSF signalling respectively revealed that the two stimuli are additive. Proliferation level 1 equates to the spontaneous level of proliferation which is reduced to level 2 in the presence of imatinib. Note that addition of GM-CSF $\geq$ 300pg/ml augments proliferation, and in the presence of imatinib, restores proliferation to the spontaneous level.

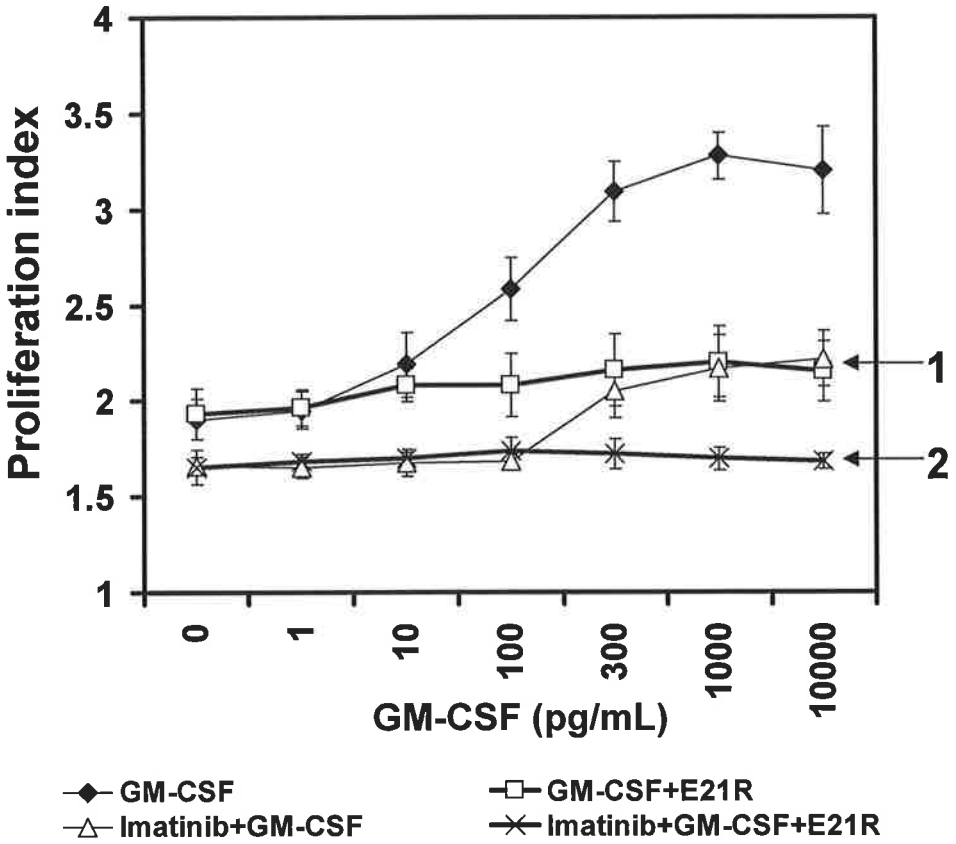

Proliferation index
4
3.5
3
2.5
2
1.5
1
0
1
10
100
300
1000
10000
GM-CSF (pg/mL)
1
2
GM-CSF
GM-CSF+E21R
Imatinib+GM-CSF
Imatinib+GM-CSF+E21R

Figure 4.8

Figure 4.9. Colony assays for the short-term fluid cultures showed the protective effect of GM-CSF in fluid culture in the presence of imatinib.

Short-term cultures of CML $CD34^+$cells were done in SDM in the 24-well plates as in CFSE assays. After 3 days, cells in each well were transferred in triplicate to 1 mL colony-culture dishes, and then were incubated for 14 days at 37 °C in a 5%CO_2 humidified-incubator. The percent of recovery colony is calculated by the ratio of colony count to the mean colony count of the pre-culture control. GM-CSF can markedly protect progenitor cells even at high concentration of imatinib resulting in little or no colony recovery from the cultures with no GM-CSF,

In addition, the result confirmed that the CML $CD34^+$ cells were not fully cytokine independent because there was significant loss of colony forming ability after 3 days in SDM. Data is also shown in tabular form in Table 4.3.

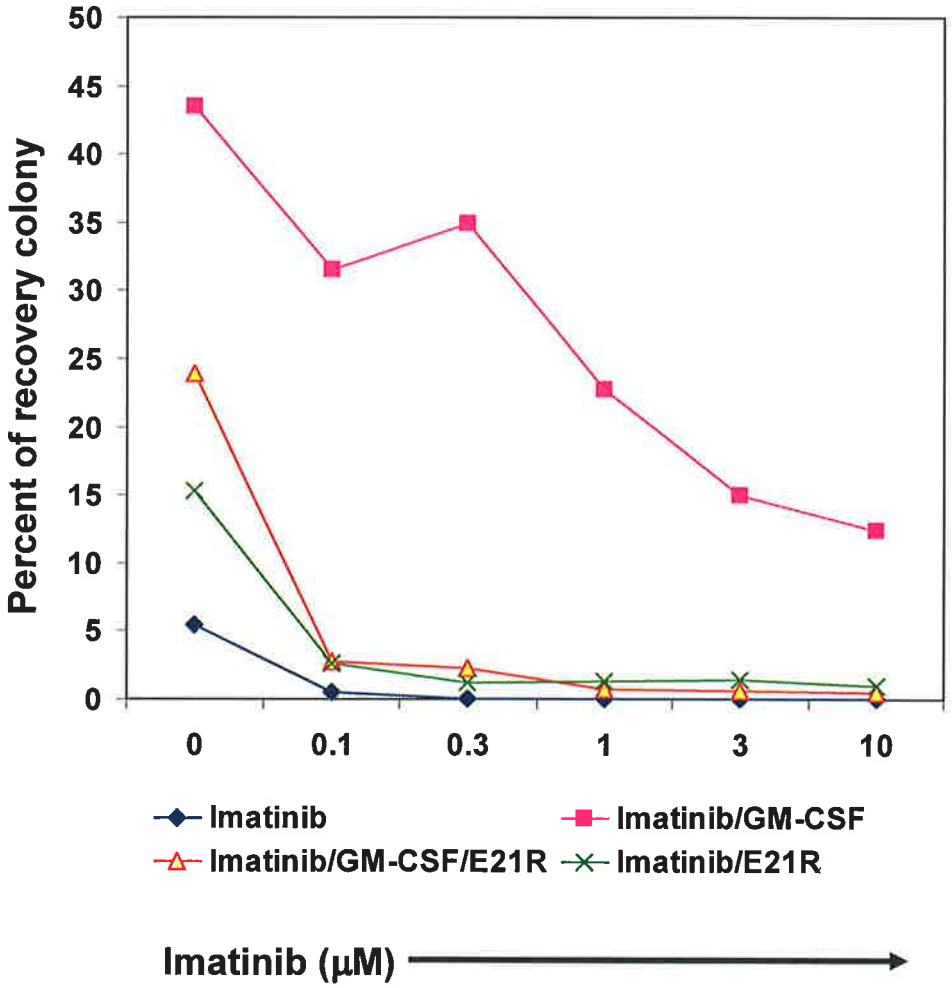

Percent of recovery colony
50
45
40
35
30
25
20
15
10
5
0
0
0.1
0.3
1
3
10
Imatinib
Imatinib/GM-CSF
Imatinib/GM-CSF/E21R
Imatinib/E21R
Imatinib (μM)

Figure 4.9

Table 4.4: Percent of recovery colonies after 3 days of culturing of Figure 4.9

Dose of imatinib (μM)	Imatinib	Imatinib + GM-CSF	Imatinib + GM-CSF+ E21R	Imatinib+ E21R
0	5.35±0.51	43.46±7.44	23.79±4.98	15.3±5.14
0.1	0.51±0.41	31.43±4.24	2.70±0.087	2.57±0.07
0.3	0	34.87±14.44	2.21±0.128	1.14±0.14
1	0	22.66±11.5	0.67±0.19	1.26±0.11
3	0	14.91±2.5	0.6±0.18	1.35±0.39
10	0.027±0.027	12.35±2.94	0.47±0.12	0.92±0.49

Control pre-culture colony forming cell number = 729.08 ± 43.8

CML $CD34^+$ cells were cultured at 50,000 cells / well for 3 days in SDM ± GM-CSF ± E21R and ± imatinib at concentration of 0, 0.1,0.3, 1, 3 and 10 μM. The total contents of each well were removed and assays for colony forming cells as described in the methodology. The percent of recovery colonies in the table arose from the ratio between colonies of conditioned wells and control pre-culture colonies.

The pre-culture content of colony forming progenitors was determined by assay of 50,000 cryopreserved CML $CD34^+$ cells.

absolute number of viable cells was the final balance between of cell proliferation and apoptosis occurring under each culture condition. Cells could have a high proliferation index but not high viability due to the effect of cell overgrowth and treatment. Therefore, to evaluate multiple parameters required identifying the time-point which was generally suitable for all parameters, particularly in the study of cell culture model lacking stimulators. Therefore, day 3 was chosen to evaluate cell culture in SDM because it avoided the masking effect due to the overall decrease of cell viability after 4 days of cultures.

Recently, Dorsey *et al* 2002 showed that IL-3 protected BCR/ABL transformed cell lines from apoptosis induced by imatinib treatment and a range of other tyrosine kinase inhibitors(Dorsey, *et al* 2002). In addition, Keeshan *et al* 2002 showed that high BCR/ABL expression levels prevented translocation of Bax and Bad to mitochondria in response to etoposide, preventing apoptosis(Keeshan, *et al* 2002). As GM-CSF was previously reported to be produced by CML cells (Jonuleit, *et al* 1998), we wished to examine the ability of this cytokine to protect cells from the action of imatinib. The ability of GM-CSF to promote additional proliferative activity to that induced by the tyrosine activity of BCR/ABL as shown in Chapter 3 supports the concept that CML progenitors are not maximally stimulated by BCR/ABL and retain responsiveness to cytokines, and GM-CSF should have a function similar to IL-3.

Imatinib was effective to inhibit cell proliferation of primary CML $CD34^+$ cells. GM-CSF could replace the BCR/ABL signaling at each concentration of imatinib used (Figure 4.3, panel a). Even at the highest level of imatinib of 10 μM, which reduced proliferation, the addition of GM-CSF raised the proliferation index to the base-line level seen when cells were cultured in SDM only (Figure 4.3, panel a., dash line). The addition of E21R fully blocked GM-CSF enhancement of proliferation. In order to complement our CFSE analysis, the colorimetric proliferation assay based on MTS reduction (Figure 4.4) which confirmed that GM-CSF significantly reduced the effect of imatinib on CML $CD34^+$ cells, supporting the concept that signaling via the GM-CSF receptor protected primary CML $CD34^+$ cells from imatinib.

The conversion of absolute number of cells to the parent cells aims to evaluate cells without division in terms of recovery of parent cells after treatment as shown in Figure 4.5 – 4.7. The normalization of data by substraction of the y-axis value at maximal dose of imatinib out of other y-axis values aims to clarify the modulation of cells to imatinib when treatments other than imatinib were added to cultures. GM-CSF maintained viability of parent cells in all patients. Particularly in patient 1, GM-CSF could maintain the recovery of parent cells to nearly the same level at any concentration of imatinib suggesting imatinib had less effect on parent cells in the presence of GM-CSF compared with other conditions. This was confirmed by the normalized data that GM-CSF could raise the IC50 of imatinib effect on parent cells. This suggested GM-CSF may partially activate the same signals as by BCR/ABL and may activate additional signals unrelated to BCR/ABL, due to the BCR/ABL blockage imatinib was maintain in culture all the time.

For the other two patients, added GM-CSF resulted in more cell survival but did not change the susceptibility of cells to imatinib suggesting GM-CSF could not activate the same signals activated by BCR/ABL, because the cell with or without GM-CSF still have the same imatinib IC50 shown by the normalized data in Figures 4.6 and 4.7. However, GM-CSF might only activate the additional signal unrelated to BCR/ABL, because GM-CSF improved the viability of parent cells over the control with only imatinib in cultures.

In order to examine the interplay between BCR/ABL and GM-CSF signaling in more detail, GM-CSF was titrated on CML $CD34^+$ cells using fixed levels of imatinib and E21R. The PI data showed the synergy between GM-CSF and BCR/ABL signaling (Figure 4.8). This finding demonstrated that CML $CD34^+$ cells could continue their proliferation in the absence of BCR/ABL function, due to signals generated by GM-CSF. The maximal level of proliferation induced by GM-CSF whilst BCR/ABL was inhibited by imatinib was essentially identical to that induced by BCR/ABL alone.

Short term liquid culture in SDM, followed by colony formation in semi-solid agar to enumerate progenitors, has shown that CML colony forming-cells were not completely growth factor independent. Addition of GM-CSF to the cultures was clearly able to preserve CML progenitors compared with no treatment. GM-CSF was also able to protect colony-forming cells from both imatinib induced and spontaneous apoptosis, and the

specific GM-CSF antagonist markedly reduced this protection. Used alone in the pre-culture for colony assays, E21R was able to preserve a small number of colony forming cells, suggesting that in the absence of any exogenously added cytokines, E21R binding of the GM-CSFR α may be able to exert subtle signaling effects (McClure, *et al* 2001, Sjoblom, *et al* 2002). However, its effect is not as potent at preserving colony-forming cells when combined with imatinib. This warrants further investigation for its implications for the clinical use of E21R.

Chapter 5.

Effect of bystander cells on CML CD34$^+$ cell behaviour

5.1. *Introduction*

Resistance to chemotherapy is a serious limiting factor in the treatment of many kinds of cancers including haematological malignancy. The increased dosage of chemotherapy required to control the disease can no longer be tolerated by the patients. Many possible imatinib resistance mechanisms have been studied to get new approaches for CML therapy. The most prevalent is mutation of the ATP loop within the tyrosine kinase portion. In addition, the constitutive tyrosine kinase activity of BCR/ABL which was not able to suppress the apoptosis at low level of BCR/ABL protein has been studied and concluded that this might ensue in the absence of IL-3, leading to the inappropriate survival and proliferation of the cells at low cytokine concentration (Cambier, *et al* 1998). This leads to focusing on the role of cytokines. Although the role of autocrine production of cytokines in IL-3 super family is still controversial with regard to disease causation (Dorsey, *et al* 2002, Jiang, *et al* 1999, Li, *et al* 2001), the mechanism through cytokines is interesting in terms of primary resistance. To date, our knowledge of primary resistance to imatinib through cytokine mechanisms is scanty. The role of cytokines in stem cell survival without proliferation has been shown in a number of articles (Malgorzata, *et al* 1999),(Daigle, *et al* 2002, Sillaber, *et al* 2000). This is a reason why cytokines are sometimes used in the treatment protocol of acute leukaemia or transplantation.

The cytokines, particularly in the IL-3 super family, are involved in the signalling pathways which can be activated by BCR/ABL. Although imatinib is able to profoundly inhibit the tyrosine kinase activity of BCR/ABL, the progenitors of CML cells may survive imatinib in the presence of cytokines through stimulation of signalling cascades not directly inhibited by imatinib particularly by cytokines in IL-3 family. GM-CSF, a member in IL-3 super family, has already been shown in this thesis to allow CML $CD34^+$ cells to escape the effects of imatinib at levels up to 10 μM. This confirmed that after BCR/ABL was suppressed by imatinib, there were additional pathways stimulated by GM-CSF providing a similar level of proliferation/survival signalling to BCR/ABL. Importantly, some centres used GM-CSF to protect bone marrow from the toxicity of chemotherapy in bone marrow transplant protocol in order to rescue normal myeloid cells from chemo-reduction. This unwanted side issue potentially rescues the leukaemic progenitors.

However, we have known that other cells produce other cytokines including GM-CSF to protect CML cells from chemotherapy as well. The previous chapter also showed that CML CD34$^+$ cells in *in vitro* culture did not produce GM-CSF but may produce very low levels of other cytokines which were not sufficient for induction of cell proliferation. However, *in vivo* the accumulation of this small amount of cytokines may be sufficient for cell survival, especially in the context of bone marrow, where local concentration at stromal surfaces may reach higher levels.

A question remains that if the accumulation of cytokines is important for cell survival, why the majority of CML patients still had remission induced by imatinib. CML cells under this scenario should still proliferate during imatinib treatment. In the patients who had complete remission, the leukaemic clone was still found by sensitive PCR technique, suggesting tissue accumulation of imatinib may be high but the endogenous factors are sufficient to allow CML progenitors to survive imatinib. On the other hand, imatinib appears to selectively kill dividing cells, therefore, progenitors which are quiescent may have a further survival advantage.

5.2. Aims of this experiment

5.2.1. To compare the survival of CML CD34$^+$ cells and total mononuclear cells using the MTS assay.

5.2.2. To examine the contribution of non-CD34$^+$ CML cells versus CD34$^+$ cells to survival of CD34$^+$ cells in cultures with imatinib.

5.2.3. To confirm the production of GM-CSF by non-CD34$^+$ CML cells using ELISpot assay and to show if production is inhibited by imatinib.

5.3. Special Methods

5.3.1. *Colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay of cell proliferation and survival.*

This method has been described in Chapter 4, but this time 3 patients whose cells were cultured in both CD34$^+$ only cultures and mixed mononuclear cultures were compared in terms of the cell survival. Three randomised cryo-preserved CML samples from patients in chronic phase at presentation were used, thawed, then ficolled to remove dead cells. Using a magnetic separation kit (MACS CD34 Progenitor Cell Isolation Kit, Becton-Dickinson), CD34$^+$ cells were selected. The purity of the sorted-cell was more than 95% detected by flow cytometry.

Forty thousand of both CML CD34$^+$ cells and mixed mononuclear cells were used for cultures in triplicate in 96-well plates using 8 different culture conditions. Cells were cultured in 100 μL of basic medium containing SDM only or SDM plus 4HGF consisting of 10ng/mL each of IL-3, G-CSF, FL-3 ligand and TPO. Cultures were supplemented with imatinib over the range 0 to 20 μM with or without 300 pg/mL GM-CSF and with or without 10 μg/mL E21R. After 4 days, MTS was added and incubated at 37 °C for 4 hours before performing ELISA plate reader measurement of OD at 490nm. Each point in Figures 5.1 to 5.3 represented the mean value of triplicate cultures.

This was to examine whether non-CD34+ cells could survive imatinib during culture. The percent CD34$^+$ cells of CML 1-3 in mixed mononuclear cultures was varied at 36%, 37% and 11% respectively. Each point represents the mean fluorescence reduction value by MTS assay of the triplicate wells.

5.3.2. *To examine the bystander effect on CML CD34$^+$ cells using dye dilution technique.*

CML CD34$^+$ cells from 4 patients were thawed as in the general method in Chapter 2. Then the mononuclear were divided into 2 groups: the CFSE-labelled cells and non-CFSE labelled cells. Both of them were stained with PE-conjugated CD34 antibody and 7-AAD. Then both of them were sorted for CD34-PE$^+$/ 7-AAD$^-$ cells. Cultures were set up to examine the proliferation behaviour of CFSE$^+$/ CD34$^+$ CML cells in the context of non-CFSE-labeled CD34$^+$ CML cells or mixed mononuclear CML cells.

Figure 5.1. Experimental design of experiments to examine bystander cell effects on CML CD34$^+$ cell proliferation and survival.

Thawed CML cells from patients in chronic phase were labeled with CFSE and cultured overnight to stabilize. Non-CFSE labeled cells were cultured in parallel to the CFSE-labeled cells, and were used for mixed mononuclear cultures. The labeled cells were sorted for CFSE$^+$ / CD34-PE$^+$ / 7-AAD$^-$ through 40-channel on FL-1. The non-CFSE labeled cells were sorted for CD34$^+$/ 7-AAD$^-$ cells. The bystander experiment was performed by mixing the sorted CFSE-labeled CD34$^+$ cells with the non-CFSE labeled cells which were either purified CML CD34$^+$ cells or total mononuclear CML cells. In this way, the proliferation and survival of seeded CD34$^+$cells could be examined in different cell contexts.

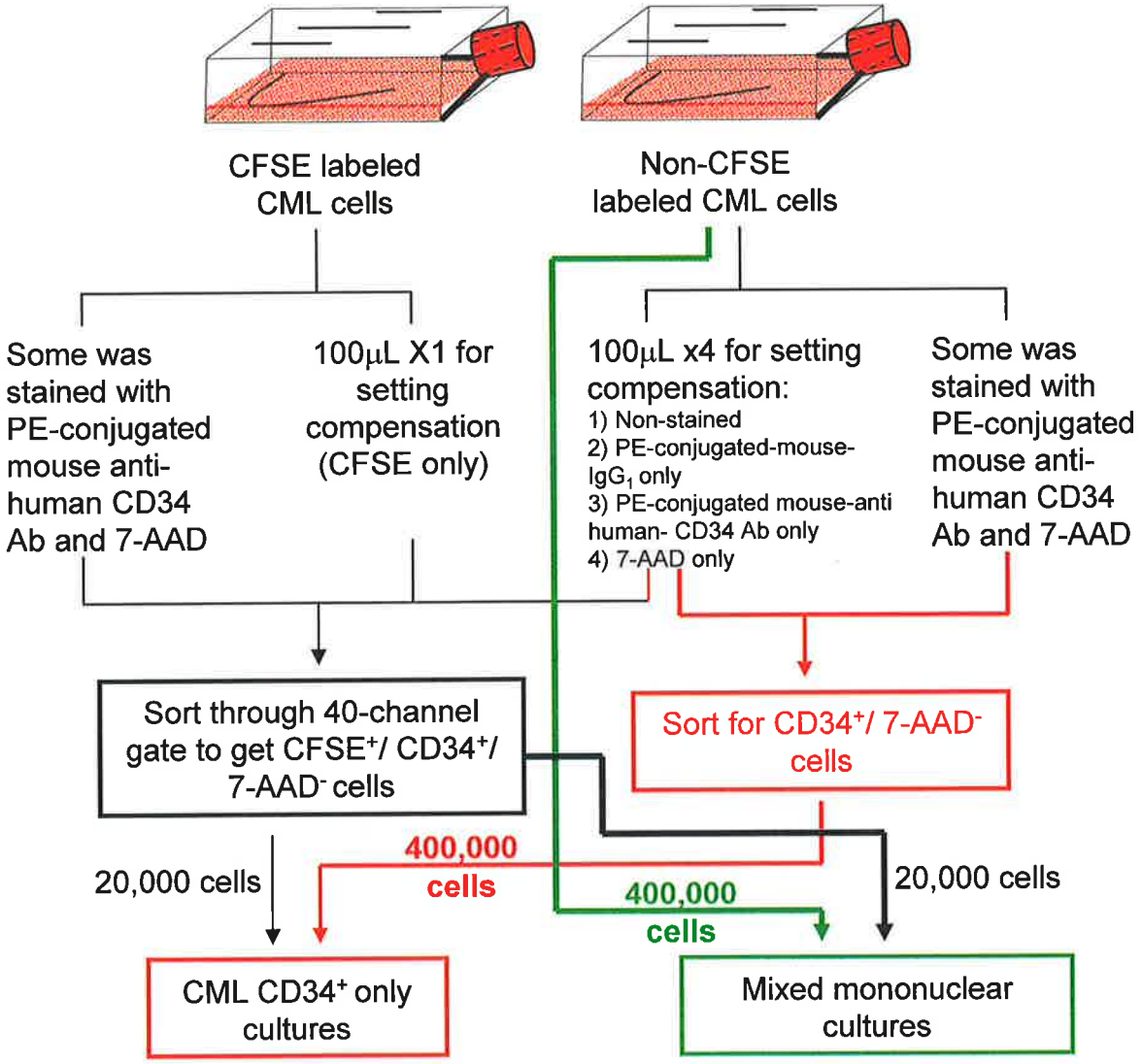

CFSE labeled CML cells
Non-CFSE labeled CML cells
Some was stained with PE-conjugated mouse anti-human CD34 Ab and 7-AAD
100μL X1 for setting compensation (CFSE only)
100μL x4 for setting compensation:
1) Non-stained
2) PE-conjugated-mouse-IgG1 only
3) PE-conjugated mouse-anti human- CD34 Ab only
4) 7-AAD only
Some was stained with PE-conjugated mouse anti-human CD34 Ab and 7-AAD
Sort through 40-channel gate to get CFSE+/ CD34+/ 7-AAD- cells
Sort for CD34+/ 7-AAD- cells
20,000 cells
400,000 cells
400,000 cells
20,000 cells
CML CD34+ only cultures
Mixed mononuclear cultures

Figure 5.1

Briefly, twenty thousand $CFSE^+$/ $CD34^+$ CML cells were seeded into 400,000 sorted non-CFSE labeled $CD34^+$ CML cells, to comprise 5% of the total population. Likewise, in separate culture 20,000 $CFSE^+$/ $CD34^+$ CML cells were seeded into 400,000 non-CFSE stained total mononuclear cells. The rational of this method is shown in Figure 5.1.

The cells were cultured in 24-well plates containing SDM (**S**erum **D**eprived **M**edium) supplemented with imatinib over the range 0 – 10 μM with or without 300 pg/mL GM-CSF and with or without 10μg/mL E21R. The cultures were incubated for 3 days in a 37 °C humidified-incubator at 5% CO_2. Cells were harvested and stained with 7-AAD and PE-conjugated CD34 antibody, and the proliferation of CFSE-labelled CML $CD34^+$ cells was examined by flow cytometry.

5.3.3. *ELISpot detection of GM-CSF production by CML cells*

5.3.3.1. *Preparation of ELISpot plate*

Elispot plates were sterilely prepared in the laminar-flow hood. Plates were carefully removed from kit-package and then pre-wet or activated with 50 μL 70% ethanol per well. After 2-minute incubation at room temperature, plates were washed 5 times with sterile water, 200μL/well. Plates were manually removed from the holder before emptying the plates. In the activation step with ethanol, the plates were not allowed to dry. If so, the activation of plates needed to be repeated before adding coating antibody. Then, 21C11 (mouse anti-human GM-CSF monoclonal antibody, IgG_{2a}, Product code: 3480-2AW-Plus, Mabtech, NSW, AU), for coating the plates was immediately added at a concentration of 15 μg/mL for 100 μL/well. Plates then were kept at 4 °C overnight before use.

5.3.3.2. *Incubation of cells in the plates*

After overnight incubation with the coating or capture antibody (21C11), plates were washed 5 times with 200μL/well sterile PBS. Then, 200 μL/well of 0.1% BSA/IMDM was immediately added to plates, and then plates were incubated for at least 30 minutes at room temperature.

Ten CML cells and three donor cells were used to culture in 96-well ELISpot plates with SDM. Briefly, five thousands of CML $CD34^+$ or non-$CD34^+$ CML cells purified by MACS and resorted by $FACStar^{PLUS}$ were added to wells, along with appropriate controls (no cells, 30 pg/mL GM-CSF). CD34 expression or non-expression was confirmed by flow-cytometry.

After cell sorting, five thousand of the required cells were re-suspended in 100 μL SDM, therefore, the final cell density was around $5x10^4$ cells/mL. One hundred μL/well of cell suspension in SDM were added in the plates after removing all 0.1%BSA/IMDM medium. Plates were wrapped in aluminium foil to prevent evaporation and incubated in a 37 °C humidified incubator with 5% CO_2 for 48 hours. During culture, plates were undisturbed to prevent movement of cells.

5.3.3.3. *Detection of GM-CSF production*

After 2 days incubation, wells were processed to detect GM-CSF production. Plates were emptied by removing the cells and washing the plates with 200 μL/well of PBS for five times. Then, 100 μL/well of 1μg/mL 23B6-biotin (biotinylated mouse anti-human GM-CSF monoclonal antibody, IgG_{2a}, Product code: 3480-2AW-Plus, Mabtech, NSW, AU), as the detection antibody, was immediately added to the empty wells. After that, plates were incubated at room temperature for 2 hours. The detection antibody (23B6-biotin) was diluted to the concentration of 1μg/mL in PBS containing 0.5% fetal calf serum (FCS). After incubating plate with 23B6, plates were washed 5 times with PBS as above, and 100 μL of Streptavidin-alkaline phosphatase at the dilution of 1:1000 in PBS-0.5%FCS was added per well, and incubated at room temperature for 1 hour. Plates were washed 5 times with PBS, and 100 μL/well of the filtered substrate solution (BCIP/NBT-plus, Product code: 3480-2AW-Plus, Mabtech, NSW, AU) was added to the plates to develop the spots on the membrane in the wells. BCIP/NBT-plus substrate solution should be filtered through a 0.45 μm-filter before use. The distinct spots in positive wells became visible after 20-40 minute-incubation at room temperature, and at this point, the reaction was stopped by washing plates extensively in tap water.

Blue/black spots due to GM-CSF production by individual cells were visualized, recorded and counted using a dissecting microscope and camera (Olympus SZ60 microscope and Olympus DP11 digital camera, Japan).

5.4. *Results*

5.4.1. *Three CML patients' cells were examined using MTS assay and found CML CD34$^+$ cells have different responses to cytokines from the CML mononuclear cells.*

In all panels a of Figure 5.2 – 5.4, considering CML CD34$^+$ only cultures, SDM supplemented with 4HGF raised the overall base line of cell survival over the SDM-only cultured cells. CML CD34$^+$ showed a reduction in cell survival over the titration of imatinib in both SDM only cultures and in SDM+4HGF cultures. Adding GM-CSF enhanced cell survival compared with the cultures without GM-CSF. Although the effect of GM-CSF on CML CD34$^+$ cells of CML 2 was not as profound as the other patients' cells and showed only minor enhancement of OD value by MTS assay compared to other conditions in the SDM plus 4HGF based group, the added GM-CSF still provided the highest survival compared with the other conditions. These findings were compatible with the data in Chapter 4 on both MTS and dye dilution assays.

In contrast, for the total or mixed mononuclear cultures, the cells showed a lot more variation of cell survival which was very difficult to evaluate. This is the disadvantage of MTS compared with CFSE dye dilution technique. However, this assays on mixed-mononuclear culture still provided some interesting information. The arrows 1 and 2 which were shown in Figures 5.2 – 5.4 were the base-line cell survival in SDM plus 4HGF base and SDM base cultures respectively.

Figure 5.2 and 5.3, panels b, CML cells of both patients had the same percentage of CD34$^+$ component of about 40% but showed the difference variation in base-line survival between SDM and SDM plus 4HGF. The survival of mixed cells of both patients still could be suppressed by imatinib. Both GM-CSF and 4HGF could protect the effect of imatinib on these cells. What was hard to know was whether the greater cell survival by 4HGF and GM-CSF come from more non-CD34$^+$ cells survive in cultures with cytokines or more

Figure 5.2. CML 1 showed the dilution effect by non-CD34$^+$ cells could change the figure of cell survival of CML CD34$^+$ cells using MTS assay.

Forty thousand cells were seeded in 100 μL SDM and SDM plus 4F in the 96-well plates. The top panel was the data from CML CD34$^+$ cells only, and the lower panel was the data from mixed mononuclear cells containing both non-CD34$^+$ CML cells and CML CD34$^+$ cells. The cultures were performed in 4 days under 8 conditions as indicated. The 4F contained 10 ng/mL each of IL-3, G-CSF, Fl-3 ligand and TPO. After 4 day cultures, 20 μL MTS was added to each well and incubated at 37°C in the dark for 4 hours before performing the plate-reader at 490 nm. Panel a showed MTS assay data of the CML CD34$^+$ cells, and panel b showed the MTS assay of the mixed mononuclear cultures.

Panel b. had 36% CD34$^+$ cells. Although the maximal survival of cells in panel b. was lower than in panel a. The base-line survival of cells in Panel b (arrow 2). became higher. If the mature mononuclear cells were not active, then the survived cells left to metabolize the formazan complex to express the fluorescence color should be CD34$^+$ cells. The change of OD value in panel b. should come from the change of survival of CML progenitors.

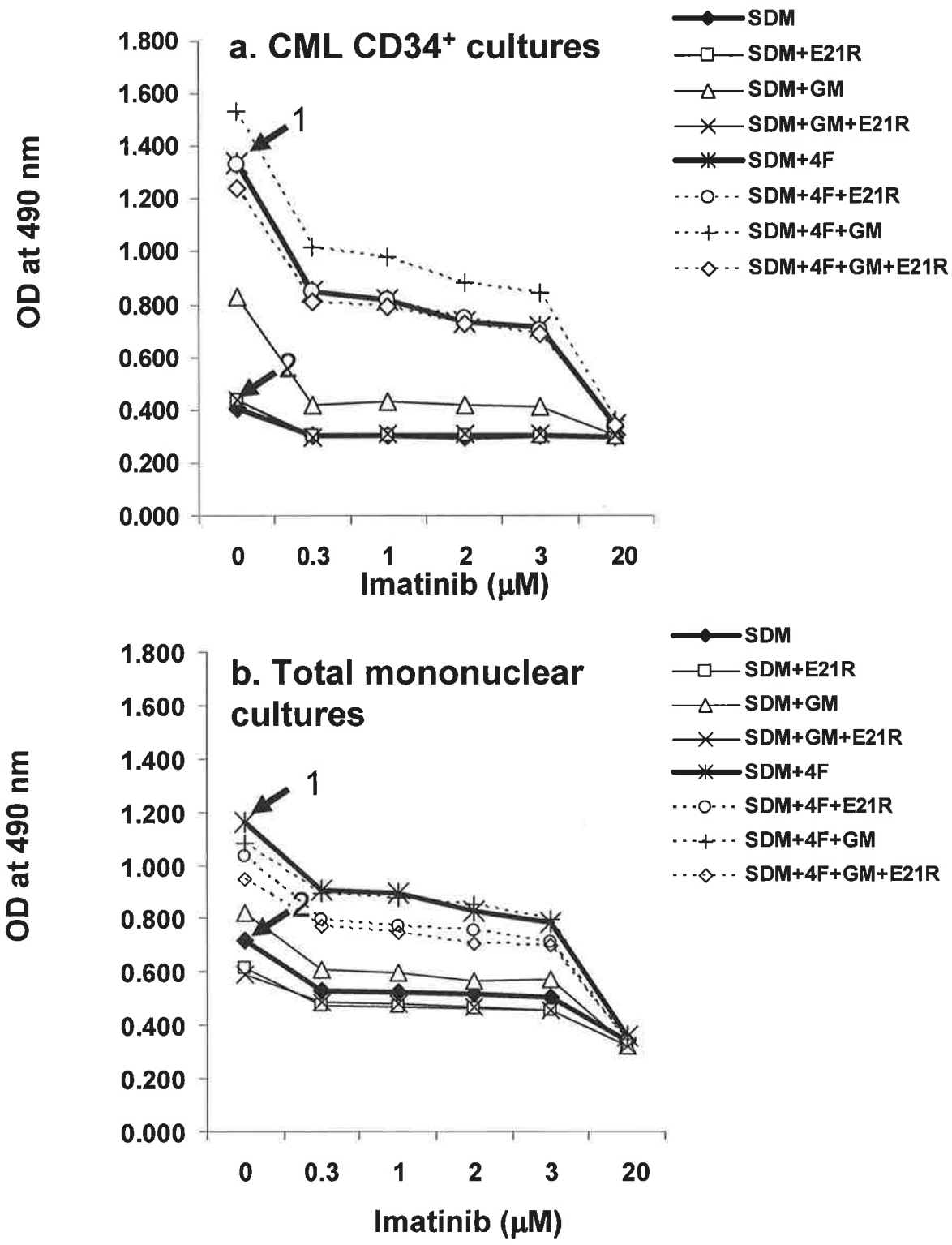

a. CML CD34+ cultures
OD at 490 nm
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
0.3
1
2
3
20
Imatinib (μM)
1
2
SDM
SDM+E21R
SDM+GM
SDM+GM+E21R
SDM+4F
SDM+4F+E21R
SDM+4F+GM
SDM+4F+GM+E21R
b. Total mononuclear cultures
OD at 490 nm
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
0.3
1
2
3
20
Imatinib (μM)
1
2
SDM
SDM+E21R
SDM+GM
SDM+GM+E21R
SDM+4F
SDM+4F+E21R
SDM+4F+GM
SDM+4F+GM+E21R

Figure 5.2

Figure 5.3. CML 2 had the same % of $CD34^+$ cells in mixed mononuclear cultures as CML1 but had a higher base-line survival.

The responses of CML $CD34^+$ cells of CML 2 to GM-CSF both in SDM only and SDM plus 4F (Panel a.) were in the same pattern as CML1. This pattern of responses was also seen in mixed mononuclear cells with SDM-only base medium. The higher base-line OD value (bold line) was also observed in panel b.

Notably, the base-line survival of cells cultured in SDM plus 4F as shown by the comparable arrows in panels a. and b.were found higher in panel b. suggesting that CML $CD34^+$ cells in mixed mononuclear cultures responded very well to the effect of non-$CD34^+$ cells and allowed the number of $CD34^+$ cells surviving to complement of non-cycle active mature cells. However, the cells which survived conditions in mixed mononuclear cultures may be only or mostly non-$CD34^+$ but not only $CD34^+$ cells because the pattern was different from the cells in $CD34^+$ only cultures.

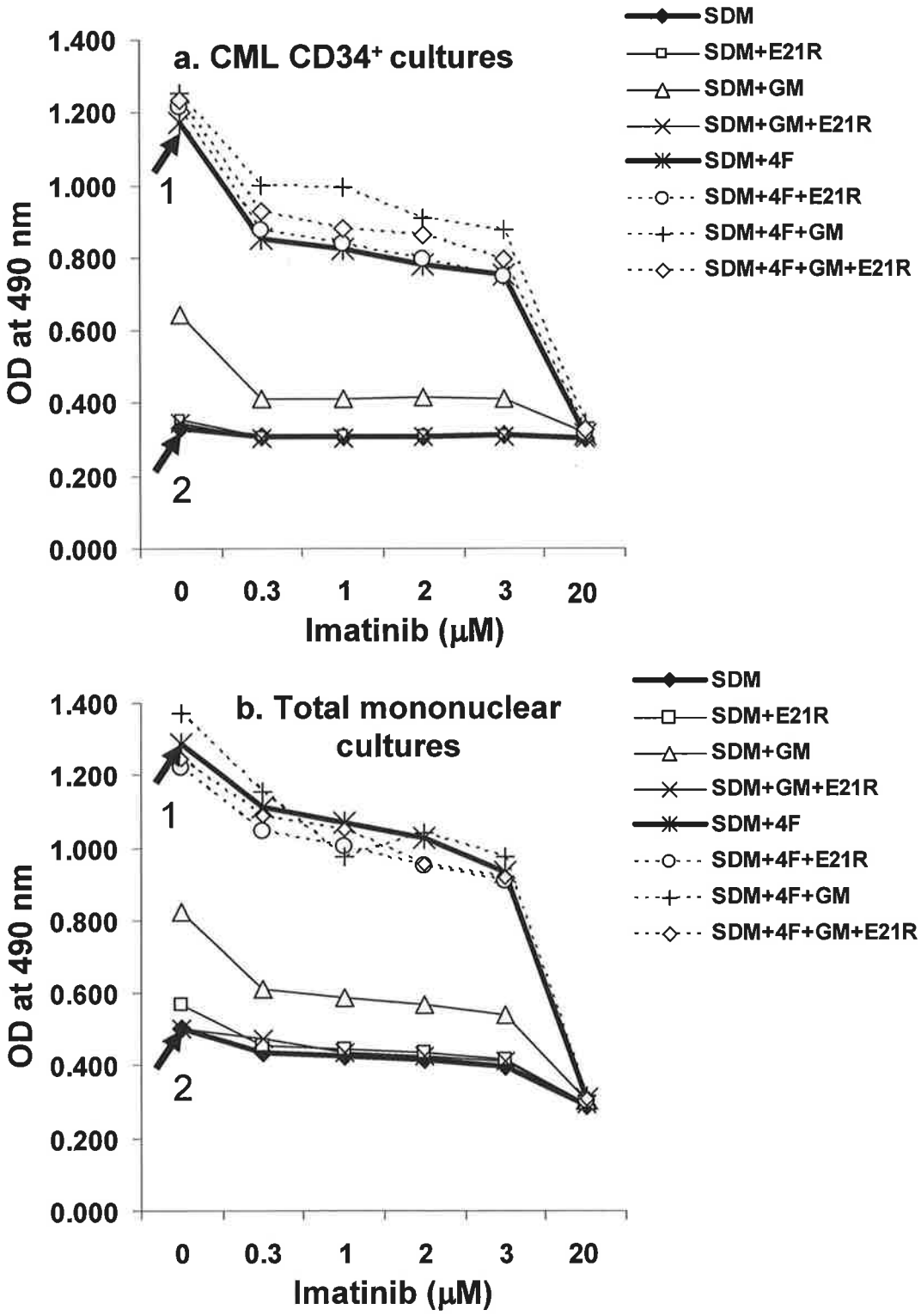

a. CML CD34+ cultures
OD at 490 nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
0
0.3
1
2
3
20
Imatinib (μM)
SDM
SDM+E21R
SDM+GM
SDM+GM+E21R
SDM+4F
SDM+4F+E21R
SDM+4F+GM
SDM+4F+GM+E21R
b. Total mononuclear cultures
OD at 490 nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1
2
0
0.3
1
2
3
20
Imatinib (μM)
SDM
SDM+E21R
SDM+GM
SDM+GM+E21R
SDM+4F
SDM+4F+E21R
SDM+4F+GM
SDM+4F+GM+E21R

Figure 5.3

Figure 5.4. CML 3 which had very low percent $CD34^+$ cells in mononuclear cultures showed no difference of cell survival among the different treatments.

Panel a, CML $CD34^+$ only culture showed a high response to GM-CSF whilst the control(non-treated cells) has very low cell survival. The imatinib has little effect on these cells. The effect of GM-CSF could promote cell survival to the same level as cells in SDM with 4F.

Panel b, the mixed mononuclear culture comprised of a low percent of CML $CD34^+$ cells (~ 10%) helped to exclude the effect of $CD34^+$ cell activity on the non-$CD34^+$ cells (Panel b.). Any input treatment had no effect on the mixed cells.

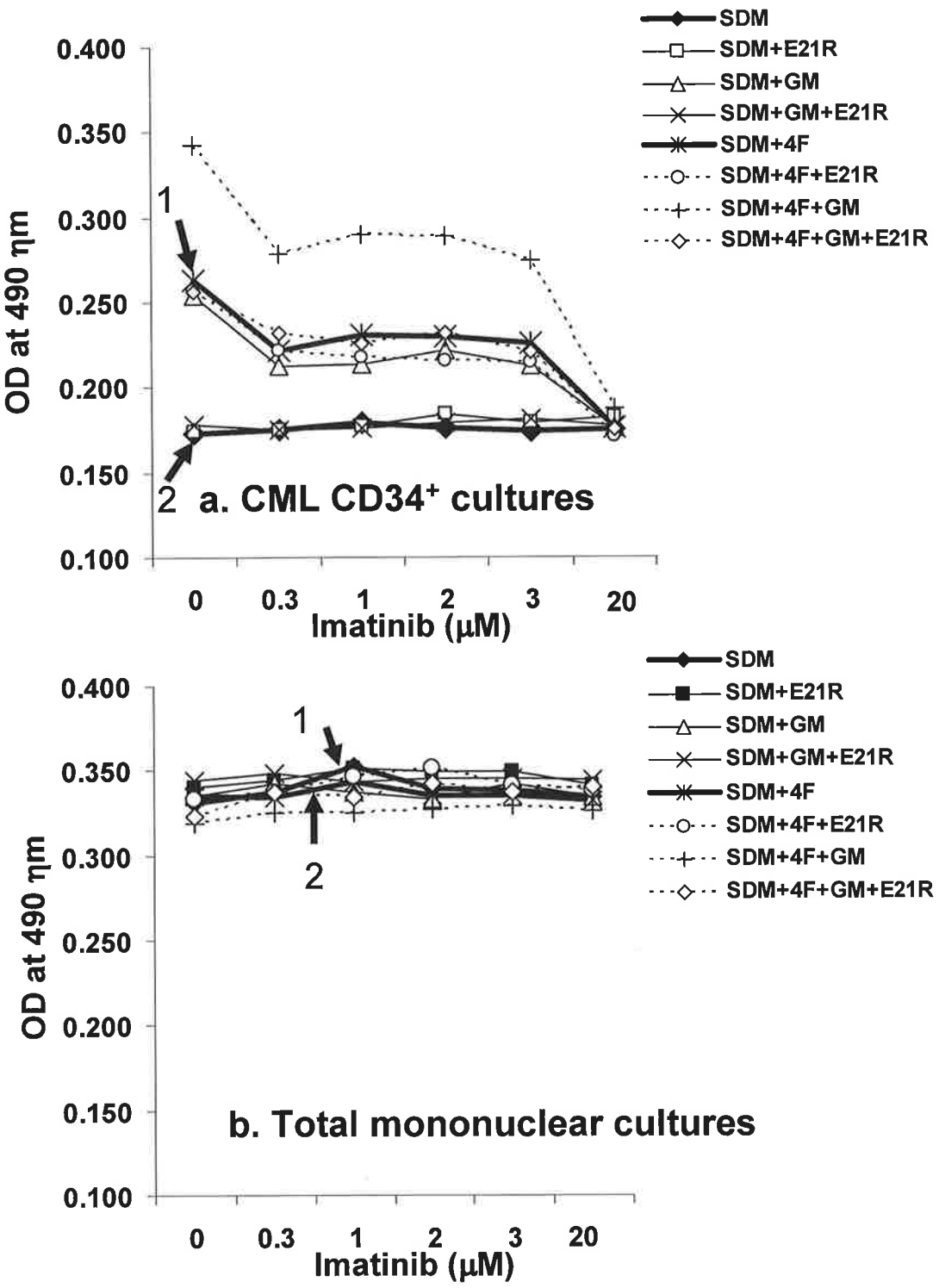

SDM
SDM+E21R
SDM+GM
SDM+GM+E21R
SDM+4F
SDM+4F+E21R
SDM+4F+GM
SDM+4F+GM+E21R
OD at 490 ηm
0.400
0.350
0.300
0.250
0.200
0.150
0.100
1
2
a. CML CD34+ cultures
0
0.3
1
2
3
20
Imatinib (μM)
b. Total mononuclear cultures

Figure 5.4

active $CD34^+$ cells were left and had their own proliferation. The non-cycling cells could not be affected by imatinib or cytokines. Therefore, a huge difference of cell survival between non-treated cells in SDM-base and SDM plus 4HGF base medium suggested that the huge cell survival by 4F might be from the activity of active cycling cells as well as CML $CD34^+$ cells.

Figure 5.4, panel b, the mixed CML cells had very low CD34 component only 10%. Imatinib even at 20 μM or other treatment combination did not change cell survival in the mixed CML cultures compared with CML $CD34^+$ only cultures. The base line survival of cells in total mononuclear (or mixed CML cells) cultures with SDM only was not different from cells with other treatment combinations and high enough to reach the same level of CML $CD34^+$ cells cultured with SDM plus 4F. This suggested non-$CD34^+$ cells were tolerant to treatment greater than CML $CD34^+$ cells.

5.4.2. *CFSE dye dilution showed CML $CD34^+$ cells underwent more proliferation in total mononuclear cultures compared to $CD34^+$ only cultures.*

CFSE dye dilution was used to examine selected CML $CD34^+$ cells in the cultures containing non-CFSE labeled mixed mononuclear cells and non-CFSE $CD34^+$ cells only. The number of labeled cells and total cells were fixed in all treatment conditions. The findings were that first, in the CML $CD34^+$ cell only cultures, non-CFSE CML $CD34^+$ cells (Figure 5.5, panel b.) at the same number as non-CFSE mixed mononuclear cells (Figure 5.5, panel a.) could not induce the proliferation of CFSE-labeled CML $CD34^+$ cells to the same extent seen in the labeled cells in mixed mononuclear cultures. Most final cultured cells were still CML $CD34^+$ cells without differentiation. Second, most of CML $CD34^+$ cells of this patient died during cultures if $CD34^+$ cells only were seeded, in contrast to the CFSE labeled cells in mixed mononuclear cultures. In addition, the cultures did not show much cell differentiation among culture conditions (Figure 5.6) within 3 days in both groups of cultures.

To compare the bystander effect on $CD34^+$ cells of either CML cells or normal donor cells (Figure 5.7), normal donor cells were labeled with CFSE and sorted using CD34-PE and 7-AAD as done with CML cells. The labeled $CD34^+$ cells were seeded in mixed

Figure 5.5. CML CD34$^+$ cells showed enhanced proliferation in the mixed mononuclear cultures.

Twenty thousand CFSE-labeled CML CD34$^+$ cells were seeded in a 24 well-plate in serum deprived medium and mixed with non-CFSE labeled mixed mononuclear (a.) and non-CFSE labeled CD34$^+$ cells (b.) at the total cell number 4 x 10^5 cells/ mL. After 3 days of culture the cells were harvested to evaluate the CFSE-labeled cell proliferation.

CML CD34$^+$ cells in mixed mononuclear cultures showed both enhanced proliferation and survival (panel a), compared with CML CD34$^+$ cells in CD34$^+$ cell only cultures (panel b).

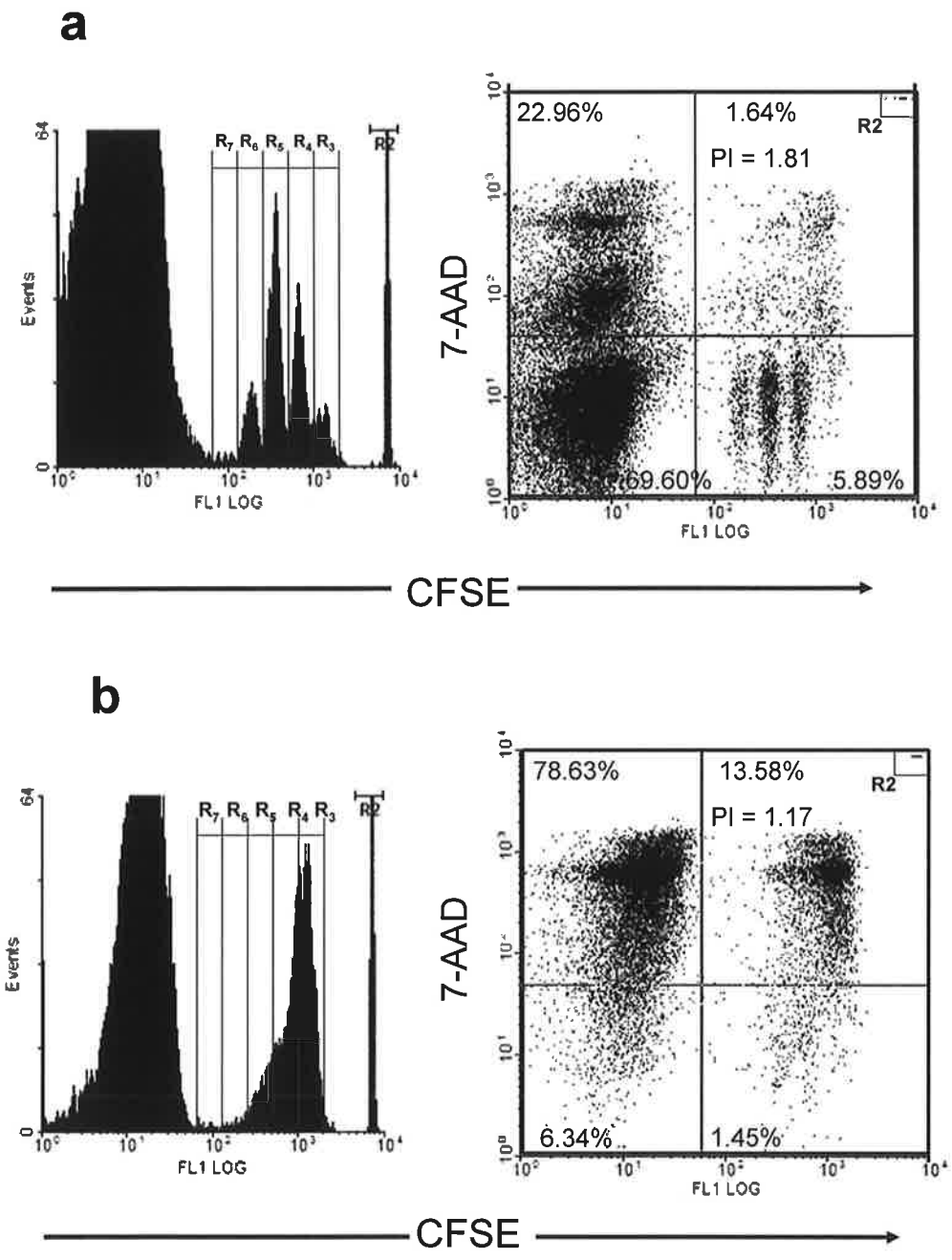

a
R7 R6 R5 R4 R3
R2
Events
FL1 LOG
22.96%
1.64%
R2
PI = 1.81
7-AAD
69.60%
5.89%
FL1 LOG
CFSE
b
R7 R6 R5 R4 R3
R2
Events
FL1 LOG
78.63%
13.58%
R2
PI = 1.17
7-AAD
6.34%
1.45%
FL1 LOG
CFSE

Figure 5.5

Figure 5.6. Cells from the same patient as Figure 5.5 was found that all CFSE-positive cells retained CML CD34$^+$ expression.

Cells from the same patients and same cultures as in Figure 5.5 were evaluated through FL1 for detection of CFSE-positive cells and FL-2 for CD34-positive cells. Sorted CD34$^+$ cells were sorted to cultures in bystander study by fixing the input cells of both total cells and CFSE-positive cells. Nearly 100% of input CFSE-positive cells were CD34-positive. Panel a and b show CFSE division profiles of CD34+ CML cells seeded into total mononuclear cell or CD34+ only cultures.

The bystander cell effect could not induce cell differentiation within 3 days. In addition, although the number of CD34$^+$ cells were equal to the number of mixed mononuclear cells in cultures, the mononuclear component rather than the CD34$^+$ component had greater effect on inducing cell proliferation than the CML CD34$^+$ cells themselves.

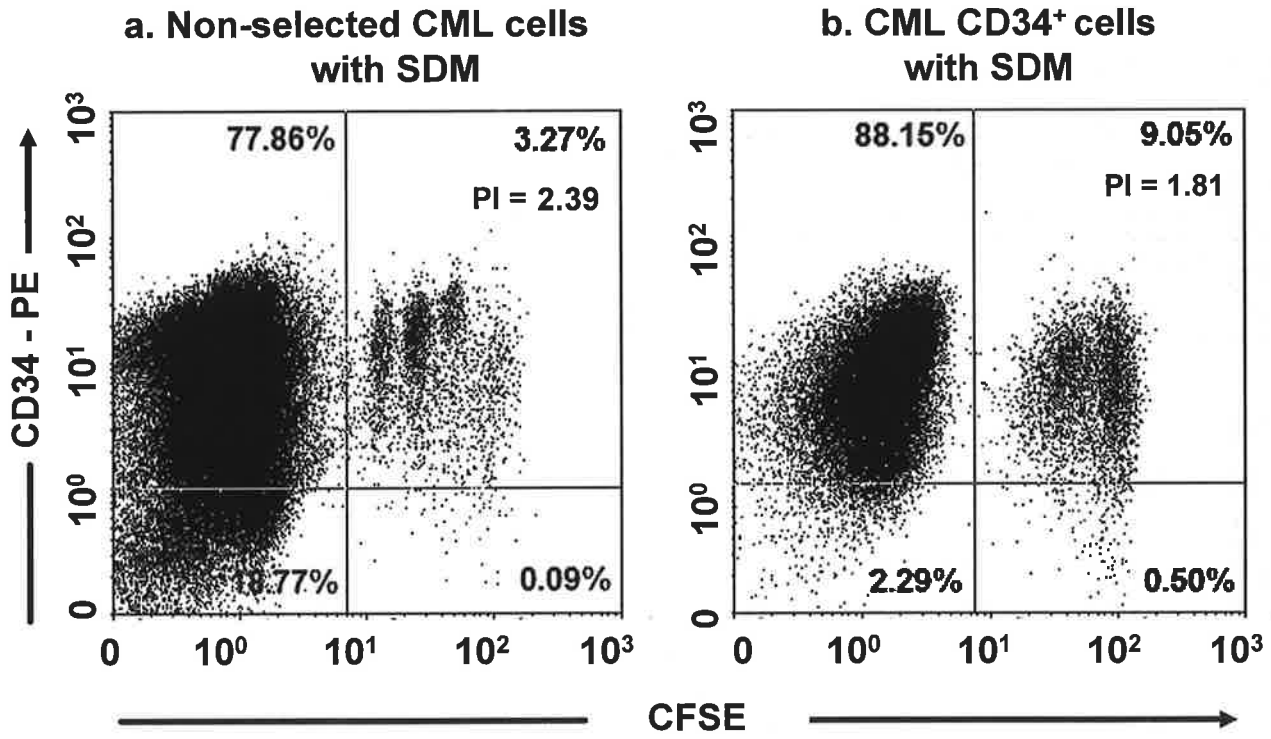

a. Non-selected CML cells with SDM
b. CML CD34+ cells with SDM
77.86%
3.27%
PI = 2.39
18.77%
0.09%
88.15%
9.05%
PI = 1.81
2.29%
0.50%
CD34 - PE
CFSE
0
10^0
10^1
10^2
10^3

Figure 5.6

Figure 5.7. CML $CD34^+$ cells showed very high levels of proliferation and survival in MNC cultures compared with the normal $CD34^+$ cells.

CML and normal $CD34^+$ cells were sorted to obtain $CFSE^+$/ $CD34^+$/ and 7-AAD^- cells as described in the methods. Sorted cells were cultured in SDM only with the bystander model to compare the proliferation activity of both types of $CD34^+$ cells. Most mononuclear cells together with CFSE-labelled $CD34^+$ cells from normal donor (panel a) could not survive in SDM as well as those from CML (panel b).

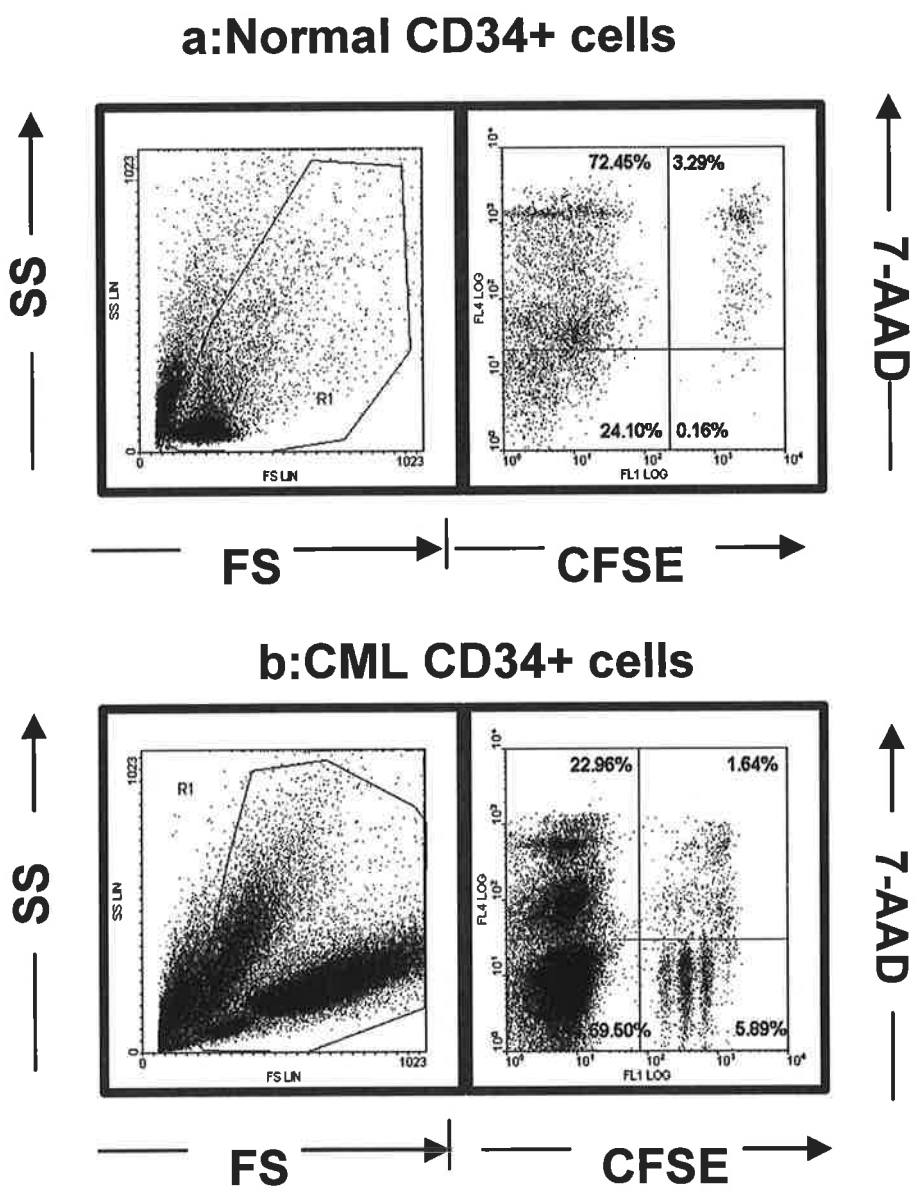

a:Normal CD34+ cells
SS
7-AAD
FS
CFSE
R1
72.45%
3.29%
24.10%
0.16%
b:CML CD34+ cells
22.96%
1.64%
69.50%
5.89%

Figure 5.7

mononuclear culture with the total cell number equal to CML cells. Cultures showed that normal CD34$^+$ cells have less proliferation activity than CML CD34$^+$ cells. Interestingly, the non-CD34$^+$ cells of CML have greater survival in culture than the normal donor cells. The cumulative cytokines produced by the normal donor bystander cells did not help donor CD34$^+$ cells to have good proliferation compared with CML cells and also did not maintain the survival of donor CD34$^+$ cells at day 3 of culture.

5.4.3. Non- CD34$^+$ *bystander cells could induce cell proliferation and viability of CML CD34$^+$ cells even at high concentration of imatinib.*

A panel of 4 chronic phase CML patients' cells was examined to determine the generality of the cell effect, and to determine the effect on imatinib responses.The labeled cells were seeded in total mononuclear cultures and CD34$^+$ cells only cultures with fixing the number of non-CFSE cells and CFSE-cells. Results from patients have been shown separately in Figure 5.8, column a. as CML CD34$^+$ only cultures, and column b. as mixed mononuclear cultures. When examining at Figure 5.8, column a. compared with b., and at 0 μM imatinib, all CML samples showed that CML CD34$^+$ cells had enhanced proliferation in mixed mononuclear cultures compared with CD34$^+$ only cultures. The proliferation of CML CD34$^+$ cells in mixed mononuclear cultures without treatment was close to the proliferation of those with GM-CSF alone in column b., and also close to the cells in CML CD34$^+$ only culture supplemented with GM-CSF only in column a.

When imatinib only was titrated over concentration of 0 – 10 μM in cultures in column a., it effectively reduced the cell proliferation about 32% (from 1.88 ± 0.38 down to 1.28 ± 0.13, Table 5.1). The added GM-CSF in CD34$^+$ cells only cultures in column a. changed the cell responses to imatinib, with the proliferation index reduction also about 31% (2.64 ± 0.23 down to 1.81 ± 0.17, Table 5.1). However, the base-line proliferation of cells treated with imatinib/GM-CSF at each point of imatinib was higher than those treated with imatinib only (column a.). The figures were different when cells were cultured in mixed mononuclear cultures. Imatinib could not reduce the base-line of proliferation of CML CD34$^+$ cells in mixed mononuclear cultures (column b.) down to the same level as those in column a.- the CD34$^+$ only cultures treated with imatinib alone. Even in non-treated cells,

Figure 5.8. The proliferation index graph derived from 4 patients confirmed that the bystander cells could protect CML $CD34^+$ cells from imatinib.

Cells from 4 patients were sorted as described in the methods to culture with SDM by being mixed with the CML $CD34^+$ cells and total mononuclear cells. The total mononuclear cells in cultures enhanced CML $CD34^+$ proliferation. Interestingly, E21R-the specific GM-CSF receptor blocking agent- could reduce the effect of bystander cells and keep cell proliferation to the same base-line as cells treated with imatinib only in the CML $CD34^+$ only cultures.

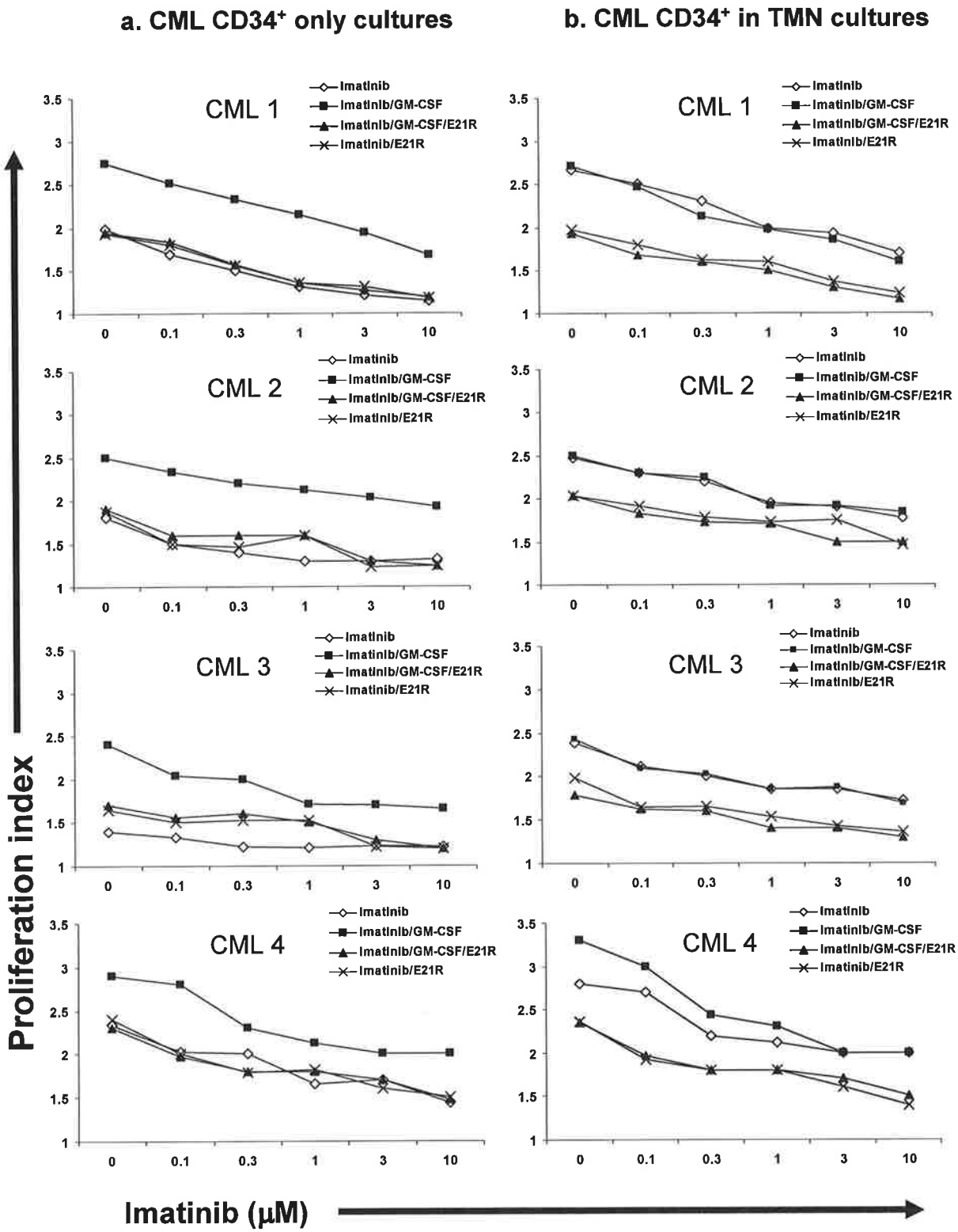

a. CML CD34+ only cultures
b. CML CD34+ in TMN cultures
CML 1
CML 2
CML 3
CML 4
Imatinib
Imatinib/GM-CSF
Imatinib/GM-CSF/E21R
Imatinib/E21R
Proliferation index
Imatinib (μM)
0
0.1
0.3
1
3
10
1
1.5
2
2.5
3
3.5

Figure 5.8

Table 5.1. The proliferation index of CML $CD34^+$ cells in $CD34^+$ only cultures

Concentration of imatinib (μM)	SDM	SDM+ GM-CSF	SDM+ GM-SF+E21R	SDM+E21R
0	1.88±0.38	2.64±0.23	1.96±0.25	1.96±0.31
0.1	1.64±0.29	2.42±0.32	1.74±0.19	1.7±0.24
0.3	1.53±0.33	2.21±0.15	1.64±0.11	1.6±0.14
1	1.36±0.19	2.03±0.21	1.56±0.19	1.6±0.19
3	1.36±0.23	1.92±0.15	1.39±0.21	1.34±0.17
10	1.28±0.13	1.81±0.17	1.28±0.13	1.28±0.15

Table 5.2. The proliferation index of CML $CD34^+$ cells in mixed mononuclear cultures

Concentration of imatinib (μM)	SDM	SDM+ GM-CSF	SDM+ GM-SF+E21R	SDM+E21R
0	2.6±0.18	2.74±0.39	2.03±0.23	2.08±0.18
0.1	2.4±0.25	2.47±0.38	1.78±0.15	1.82±0.13
0.3	2.2±0.12	2.21±0.17	1.68±0.1	1.71±0.09
1	1.98±0.11	2 ±0.2	1.6±0.18	1.67±0.12
3	1.92±0.06	1.91±0.07	1.5±0.17	1.54±0.17
10	1.79±0.14	1.78±0.17	1.37±0.16	1.36±0.09

CML $CD34^+$ cells showed higher proliferation in mixed mononuclear culture than in $CD34^+$ only culture. All CML cells had the same trends of cell proliferation in both selected CML $CD34^+$ cells and mixed mononuclear cultures. The tabulated proliferation index data at different conditions is shown in Tables 5.1 and 5.2. The data in the tables is expressed as mean ± 95% confidence interval which was derived from the mean proliferation index of 4 CML patients' cells in Figure 5.8. The mean ± 95%CI was the easy method to compare the PI of CML $CD34^+$ between the cultures with and without bystander cells.

E21R was used to examine whether it could suppress the bystander effect. The results show that E21R was able to reduce the cell proliferation down to the same level as the base-line of proliferation of CML $CD34^+$ cells in $CD34^+$ cells only cultures. This suggests that GM-CSF was the predominant cytokine produced by the bystander cells able to enhance $CD34^+$ proliferation and modulate the effect of imatinib.

5.4.4. *The effect of bystander cells was important for maintenance of cell survival rather than cell proliferation*

The same CML $CD34^+$ cells as in Figure 5.8 were evaluated in terms of the viability responses to imatinib or imatinib plus other treatment as in Figure 5.8. In Figure 5.9, CML $CD34^+$ cells had their viability reduction over the titration of imatinib from 0 – 10 μM in the cultures which contained only CML $CD34^+$ cells. GM-CSF could maintain the higher cell viability as seen in graphs in column a. which showed the similar responses in all patients. In column b., however, the level of cell viability in cultures treated with imatinib only and with imatinib+GM-CSF was the same. In addition, the overall base-line viability of CML $CD34^+$ cells from 3 in 4 patients in column b. was higher than the overall viability in column a. This is in contrast to the proliferation graphs in Figure 5.8 which showed the overall base-line PI seemed similar in both column a. and b. In Figure 5.8 and 5.9, although minimal PI of CML $CD34^+$ cells in mixed mononuclear cultures has the same proliferation indice as the minimal PI of cells in $CD34^+$ only cultures, those in mixed mononuclear cultures had higher viability than the cells in $CD34^+$ only cultures.

Figure 5.9. The bystander cells could maintain cell survival in the cultures with added imatinib.

CML cells as shown in Figure 5.8 were evaluated in terms of cell viability using 7-AAD. The percent viability derived from the ratio of 7-AAD$^-$ cells within the total CFSE-labelled cells after 3 days in SDM. The bystander within the TMN cultures could help CML CD34$^+$ cells to escape the killing of imatinib and raise the base-line of cell viability to the same level as GM-CSF treated cells. Interestingly, although E21R could block the effect of the bystander but could not bring the base-line of cell viability down to the same level as cells treated with imatinib alone in CML CD34$^+$ only cultures.

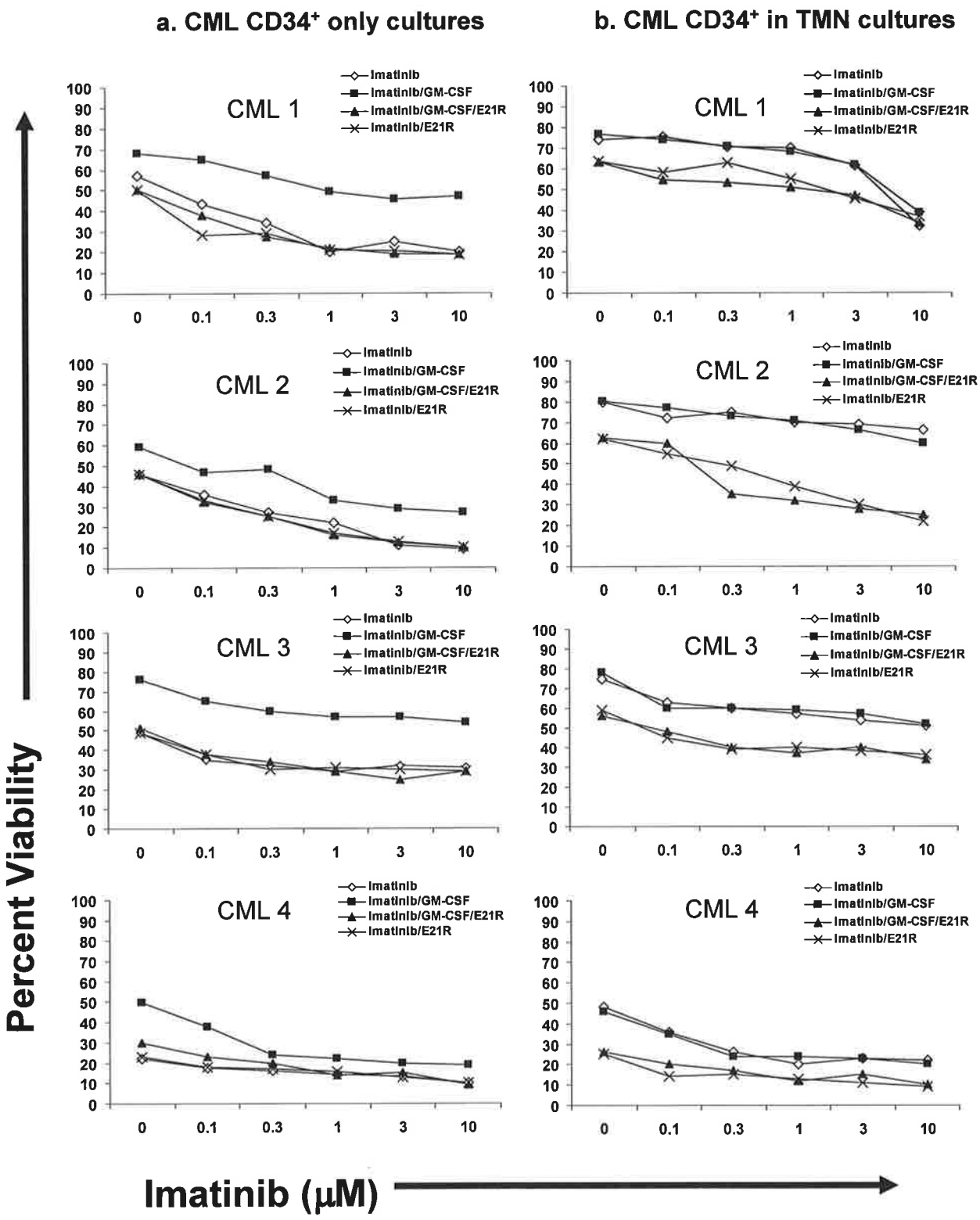

a. CML CD34+ only cultures
b. CML CD34+ in TMN cultures
CML 1
CML 2
CML 3
CML 4
Imatinib
Imatinib/GM-CSF
Imatinib/GM-CSF/E21R
Imatinib/E21R
Percent Viability
Imatinib (μM)
0
0.1
0.3
1
3
10

Figure 5.9

Table 5.3. The percent viability of CML $CD34^+$ cells in $CD34^+$ only cultures

Concentration of imatinib (μM)	SDM	SDM+ GM-CSF	SDM+ GM-SF+E21R	SDM+E21R
0	43.6±14.9	63.3±11	44.35±9.62	42.05±12.57
0.1	32.9±10.8	53.7±13	32.7±6.9	29.35±8.35
0.3	27.3±8	47.3±16	26.65±5.71	25.25±5.79
1	21.4±5.9	40.4±15.5	20.2±6.6	21.13±6.79
3	20.4±9.7	38±16.3	17.88±5.3	19.13±7.9
10	17.5±10.1	36.7±16.1	16.68±9.11	16.93±8.85

Table 5.4. The percent viability of CML $CD34^+$ cells in mixed mononuclear cultures

Concentration of imatinib (μM)	SDM	SDM+ GM-CSF	SDM+ GM-SF+E21R	SDM+E21R
0	69.25±14.12	70.3±15.9	51.9±17.2	52.4±18
0.1	61.6±17.5	61.5±18.8	42.6±17.4	43.05±19.8
0.3	57.9±22	57.03±22	36.3±14.6	41.43±19.7
1	54.2±23.12	55.6 ±21.2	33±15.8	36.75±17.1
3	51.8±19.8	51.9±19.2	32.5±13.7	31.1±14.5
10	42.8±19.2	42.6±17.14	25.6±11	25.9±12.9

5.4.5. *GM-CSF was shown to be produced by non-CD34$^+$ CML cells using ELISpot assay*

Using GM-CSF blockade E21R was an indirect way to identify activity of GM-CSF produced by the primary bystander cells. To confirm the production of GM-CSF by non-CD34$^+$ CML cells, the direct detection of their production by ELISpot was used as described in the special methods. GM-CSF producing cells were much more highly represented in the non-CD34$^+$ CML cell fraction compared with the CD34$^+$ cell fraction. In Figure 5.10, the majority of CML CD34$^+$ cells had background production levels of ≤ 0.4%, apart from the level of < 4% of GM-CSF in two CML CD34$^+$ cells. Most of non-CD34$^+$ CML sorted cells had detectable proportion of GM-CSF producing cells, with 9/10 having ≥ 0.5% and 3/10 ≥ 10%. In two samples, their proportion was as high as 70-80% of input cells. In the two cases, where CD34$^+$ CML samples exhibited significant production, it is conceivable that this was due to contamination by non-CD34$^+$ cells. Titration of imatinib revealed no impact of BCR/ABL blockade in short term culture (Figure 5.11). Examination of normal bone marrow revealed that GM-CSF production was confined mostly to the non-CD34$^+$ compartment in 3/3 donors tested (Figure 5.12). Although percent of GM-CSF producing cells of donor non-CD34$^+$ cells was generally not lower than in non-CD34$^+$ CML cells, the cells in the cultures were still CML cells because the initial input cells had 80 – 90% FISH positive for BCR/ABL .

5.5. ***Discussion***

The MTS assays in this chapter aimed to examine the different cell growth pattern between CML CD34$^+$ cells and mixed mononuclear cells containing different percent of CML CD34$^+$ cells. This could not strongly show the effect of bystander cells on CML CD34$^+$ cells but the change of percent CML CD34$^+$ cells changed the cell survival in mixed mononuclear cells.

The change of cell survival in mixed mononuclear culture might be the dilution effect of non-CD34$^+$ cells in the cultures. The MTS assays could only evaluate the total surviving cells. The concept that the mature cells could not enter cell cycle helps to suggest that the mature cells at 3 day time-point had only 2 fates in cultures. Those are that they maintained

Figure 5.10. ELISpot assay demonstrates that GM-CSF is produced by non-CD34$^+$ CML cells in culture.

CML and donor CD34$^+$ and CD34$^-$ cells were seeded at 5,000 cells/well into GM-CSF ELISpot plates, in SDM only. After 2 days culture, plates were developed to reveal GM-CSF production by individual cells. The ELISpot figure has shown in Figure 5.10.1. Number labeled over 1 - 10 was the patient number. Column a is the CD34$^+$ and b is non-CD34$^+$ CML cells. The distribution of percent cells that produce GM-CSF was shown in the graph in Figure 5.10.2. One spot shown in the well represented one cell that produced GM-CSF.

This study revealed that GM-CSF producing CML cells were most common in the non-CD34$^+$ subset, and ranged from 0.5 to 80% of input cells. In contrast, most of the CD34$^+$ CML cells had fewer than 0.4% of input cells able to produce GM-CSF.

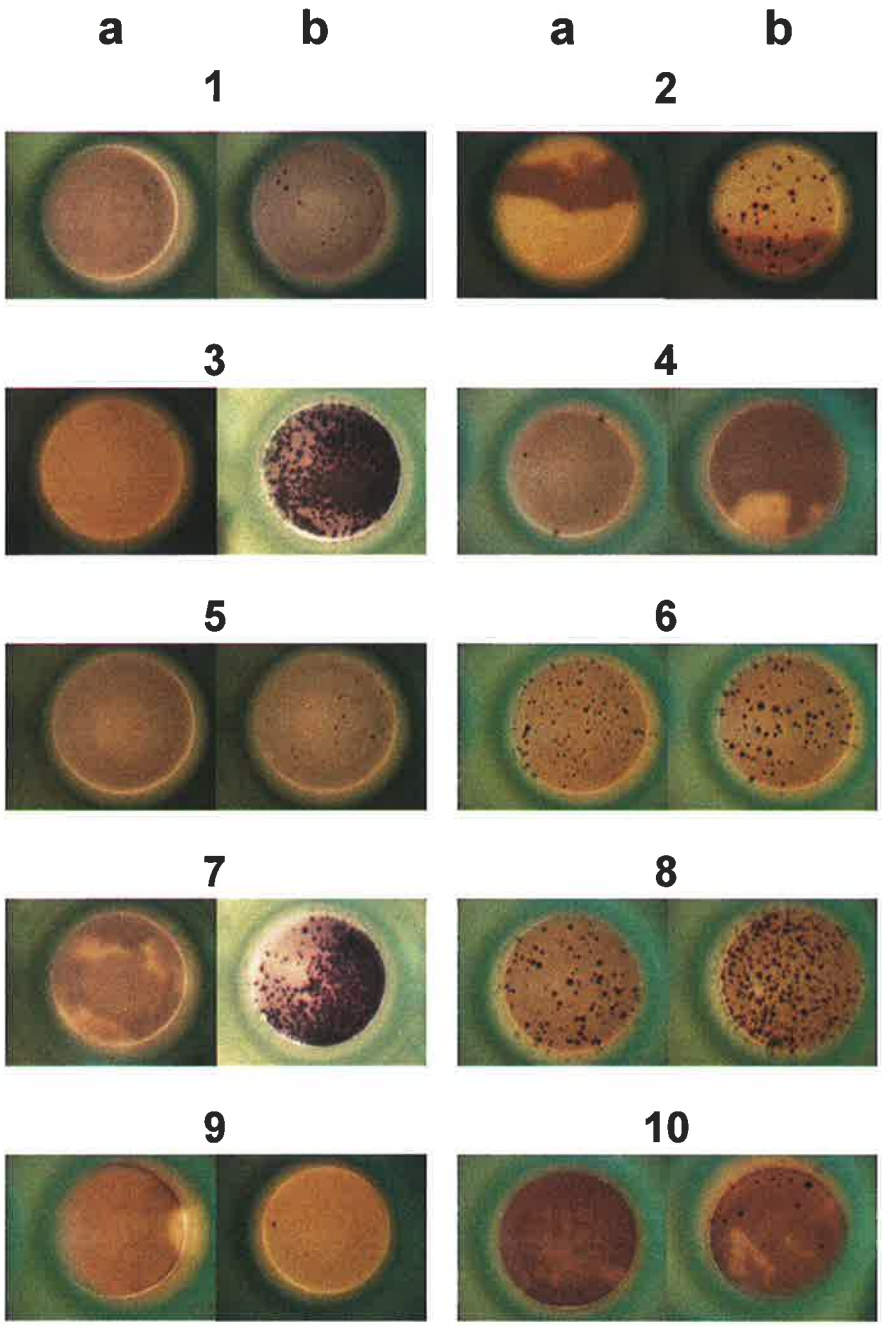


Figure 5.10.1

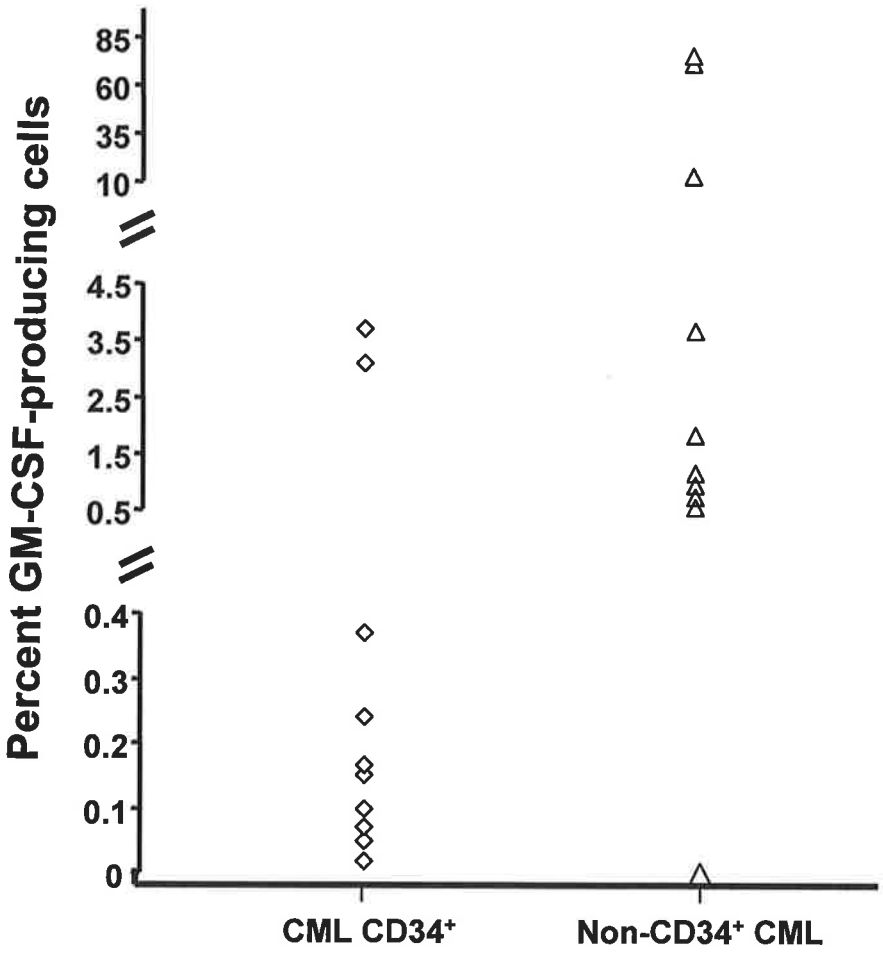

Percent GM-CSF-producing cells
85
60
35
10
4.5
3.5
2.5
1.5
0.5
0.4
0.3
0.2
0.1
0
CML CD34+
Non-CD34+ CML

Figure 5.10.2

the same number, or died (reduced survival cell number). This was supported by the findings in CFSE study on bystander cells compared with CML CD34$^+$ cells (Figure 5.5 and 5.6). The CFSE study showed that most dead cells were CD34$^+$ cells but not non-CD34$^+$ cells. Therefore, cell survival should not change much from the non-treated cultures in SDM-base medium. As seen in CML 3, Figure 5.4, panel b, the majority of cells were non-CD34$^+$ cells and were not cell-cycle active. Therefore, the 4F should not make much difference among the cultures in mixed mononuclear cultures in CML 3. Some increase of cell survival by OD value as shown in Figure 5.2 and 5.3, panel b, should mainly be arisen from the proliferation activity of CD34$^+$ component. It was not clear cut to evaluate the activity of CML CD34$^+$ cells in mixed mononuclear culture by MTS assays, but the huge change of cell survival value during treatment in CML1 and 2 could not be due to other reasons than the change of survival of cell-cycle active cells which were mainly progenitor cells. Therefore, the decrease of different intervals between cells in SDM- and SDM+4F-base in mixed mononuclear compared with CML CD34$^+$ cell cultures should be from the dilution effect by non-cell-cycle active cells. The result in CML 3 in mixed mononuclear cultures also suggested that the non CD34$^+$ CML cells could tolerate the treatment by imatinib.

CML CD34$^+$ cells have been found that GM-CSF could enhance their spontaneous proliferation and, in some CML cells, CD34$^+$ cells were also hypersensitive to GM-CSF. These findings were compatible with the previous reports examining the effect of IL-3, presumably because GM-CSF and IL-3 are in the same cytokine family group, and signals through a common β receptor. GM-CSF has been shown by Holyoake *et al* to enhance the effect of BCR/ABL, using CFSE dye dilution. Although combining GM-CSF with STI571 (imatinib) did not alter proliferation of progenitor cells in cultures with the high concentration of imatinib of 10 μM (Graham, *et al* 2002), the progenitors appear to survive without proliferation. This suggested GM-CSF may act via an anti-apoptotic effect rather than by a proliferation based counterpart.

In Chapter 4, exogenous GM-CSF was used as a model to examine the proliferation and viability of CML CD34$^+$ cells in cultures with or without imatinib. Interestingly, as GM-CSF not only enhanced cell proliferation in the presence of imatinib but also kept

Figure 5.11. The ELISpot to detect the GM-CSF producing cells showed imatinib has no effect on the production of GM-CSF in short-term cultures.

CML cells from 3 patients, number 1, number 5 and number 7 as shown in figure 5.10 were sorted to obtain the purified $CD34^+$ and non-$CD34^+$ CML cells to be seeded in the ELISpot plates and cultured in SDM supplemented with imatinib over the range 0-10 mM for 2 days as shown in Figures 5.11.1- 5.11.3. There was no visible difference in the GM-CSF producing cell number which was expressed by the black/blue dots on the ELISpot plates.

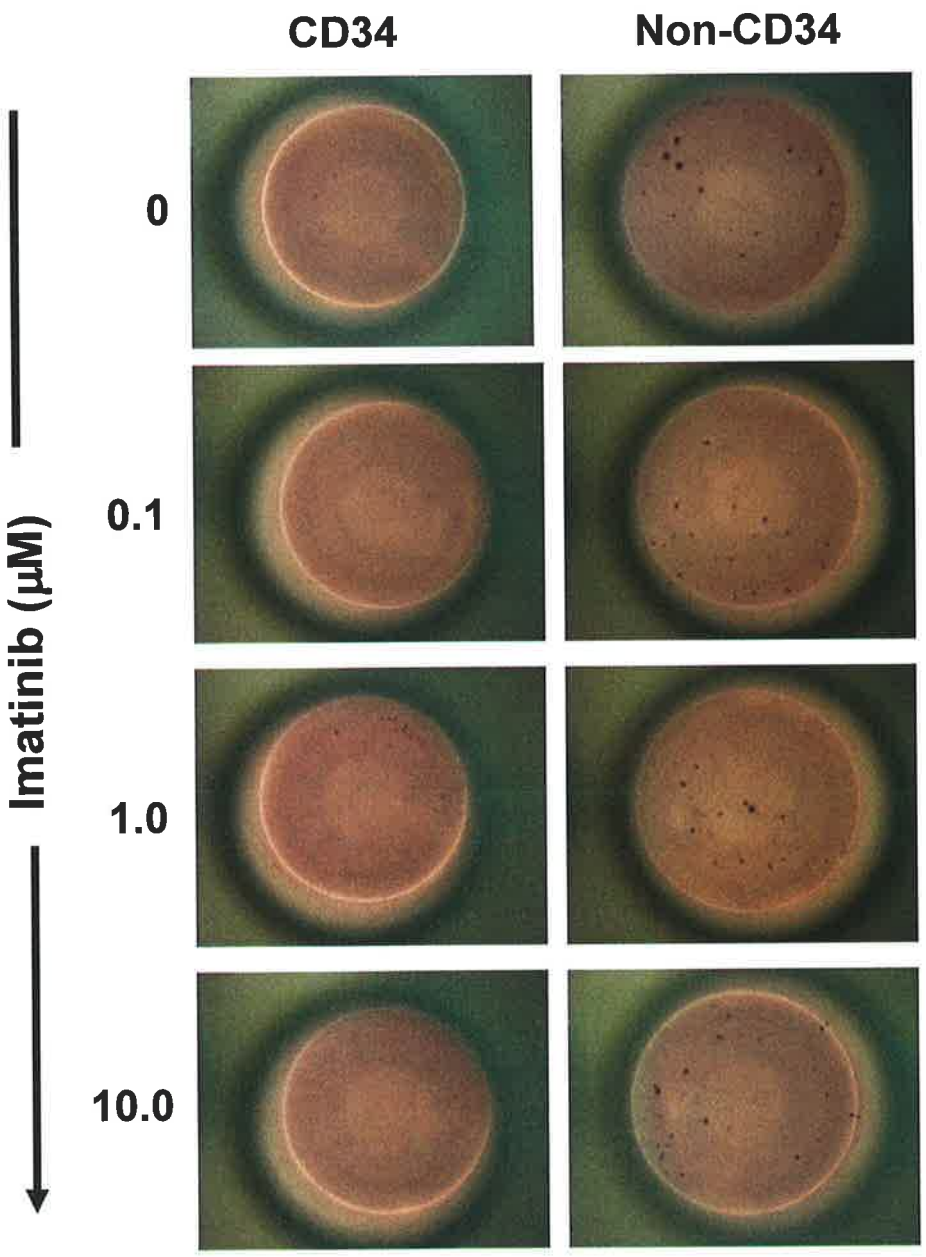


Figure 5.11.1, CML 1

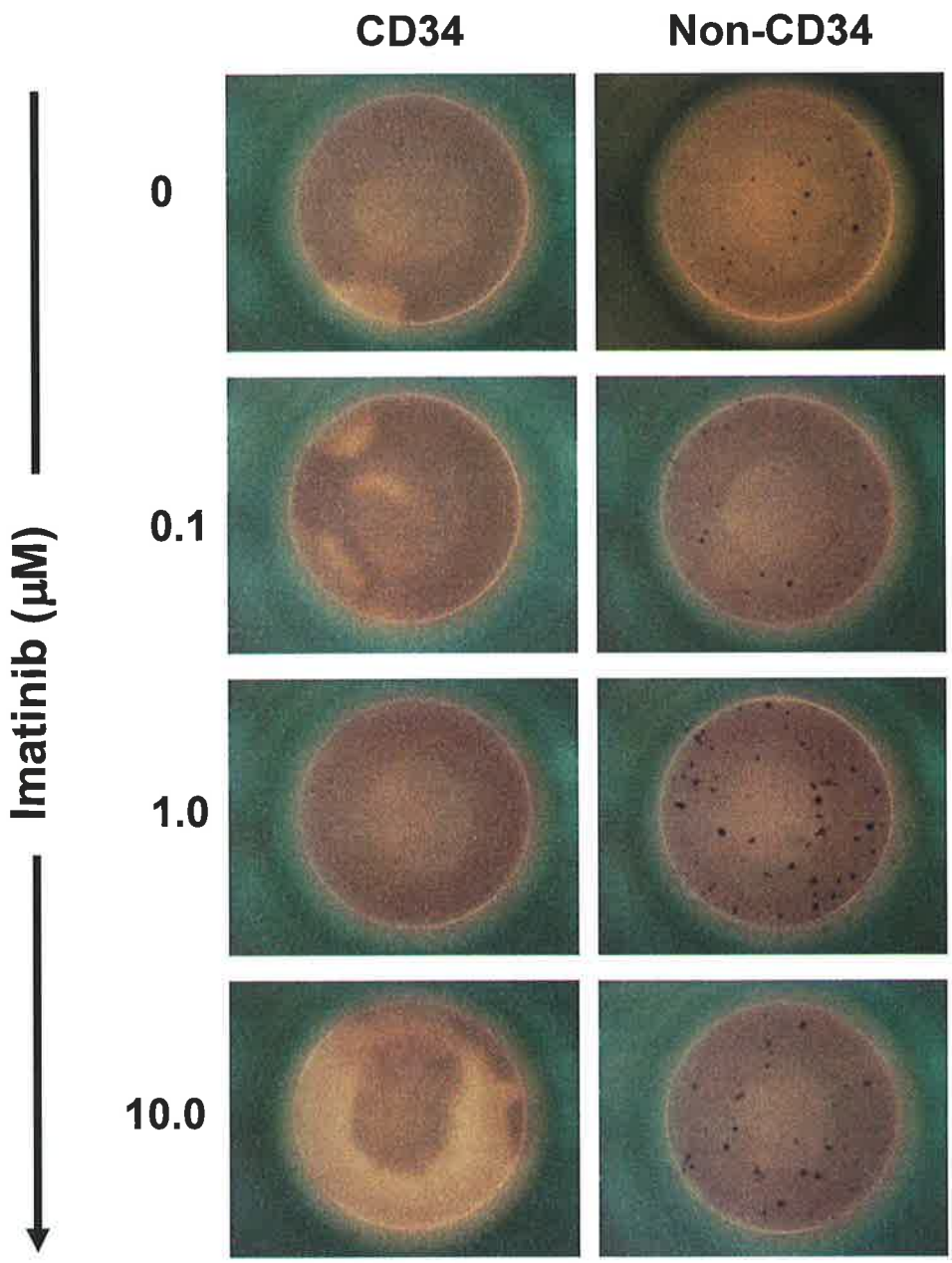


Figure 5.11.2, CML 5

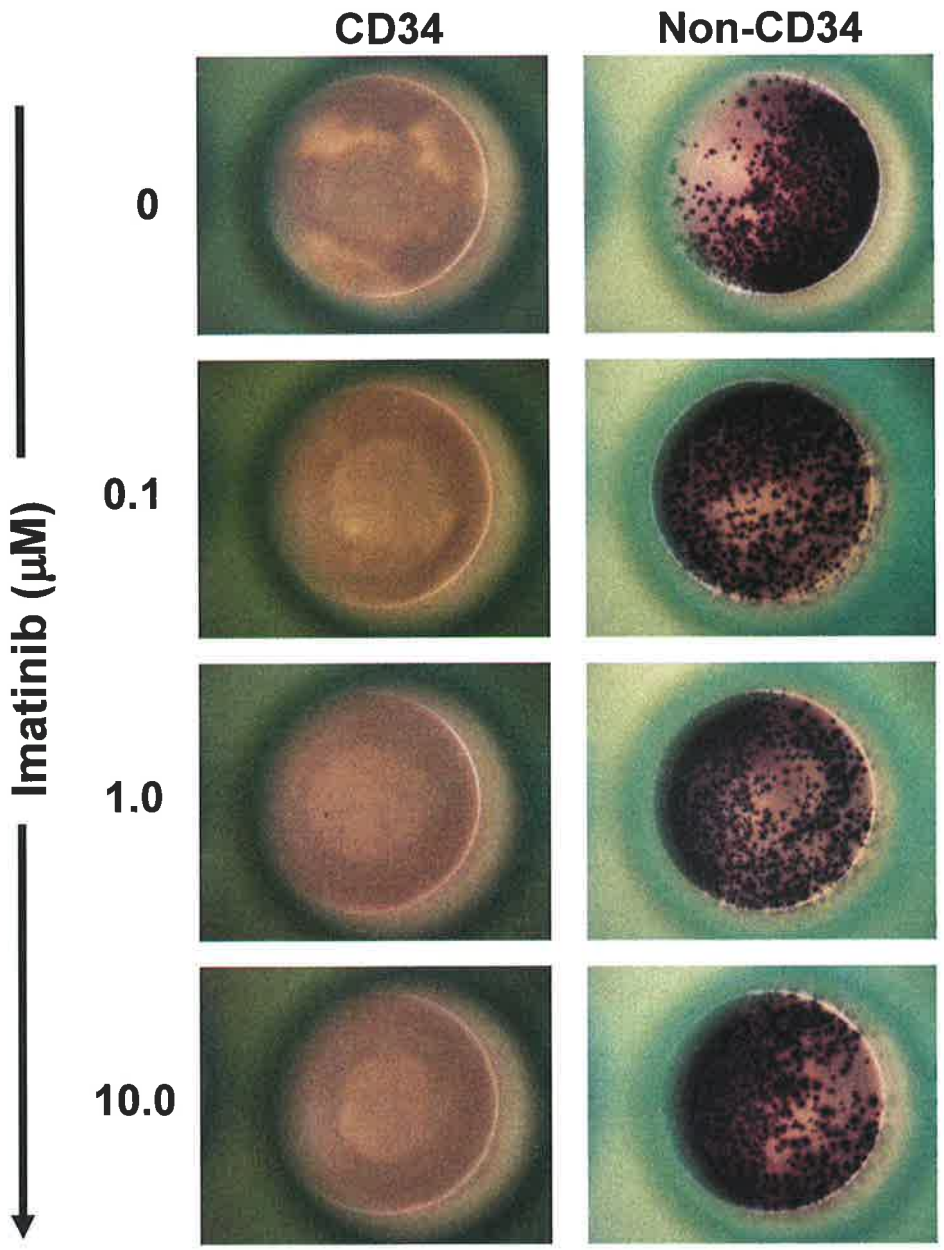


Figure 5.11.3, CML 7

progenitors alive even in cultures with high concentration of imatinib. Previous studies demonstrated that GM-CSF was found at high levels in serum of CML patients, in contrast to IL-3 and G-CSF, which was not detected(el-Ahmady, *et al* 1997, Jonuleit, *et al* 1998, Lajmanovich, *et al* 1993). Taken together, GM-CSF may be important for the protection of CML $CD34^+$ cells from the specific treatment as imatinib.

A report has been shown that IL-3 and G-CSF but not GM-CSF transcript were found in CML $CD34^+$ cells (Jiang, *et al* 1999). This is in contrast to the results in Chapters 3 and 4, we had indirectly shown that GM-CSF together with other cytokines was not produced by CML $CD34^+$ cells at the level sufficient to promote CML $CD34^+$ cell proliferation. It was because we found that GM-CSF at very low concentration could not additively enhance the function of BCR/ABL, compared with the non-treated control. This reflects that other cytokines should behave the same pattern as GM-CSF as well. This was due to the evidence that CML $CD34^+$ cells added with GM-CSF at low level and without treatment had the same proliferation idices, and CML $CD34^+$ cells needed threshold level of cytokine to overcome the effect pf BCR/ABL as found in Figure 3.5. If the cytokines are not produced by CML $CD34^+$ cells, they may be produced by the non-$CD34^+$ CML cells. Therefore the bystander experiment was performed using CFSE dye dilution technique following the diagram in Figure 5.1.

The lowest level of proliferation by CFSE labeled cells in CML $CD34^+$ only cultures (cells treated with imatinib only, Figure 5.8, column a) compared to those in mixed mononuclear cultures (cells treated with imatinib with E21R, or imatinib with E21R and GM-CSF in Figure 5.8, column b) suggested that the autocrine production of cytokines by CML $CD34^+$ cells is insufficient to result in similar proliferation level of cells without treatment in mixed mononuclear cultures shown in Column b. This is quite surprising because G-CSF and IL-3 transcript was found in the CML $CD34^+$ cells by Jiang *et al.* (Jiang, *et al* 1999). The possibility was that these cytokines were transcribed but were not excreted from the CML $CD34^+$ cells, or excreted at low levels unable to affect CML $CD34^+$ cell proliferation. This confirmed the findings in Chapter 3 and 4. In addition, the finding that E21R suppressed the proliferation of CML $CD34^+$ cells in mixed mononuclear cultures (Figure 5.8, colum b) down to the same level as cells in $CD34^+$ only cultures containing

Figure 5.12. Normal donor cells showed that GM-CSF production was confined predominantly to the non-CD34$^+$ CML cells.

Three normal donor cells have been sorted to obtain the purified CD34$^+$ and non-CD34$^+$ cells. After 2 days of cultures with SDM in the ELISpot plate, the developed black/blue dots representing the GM-CSF-producing cells showed the prominent number in the non-CD34$^+$ cells as seen in CML cells.

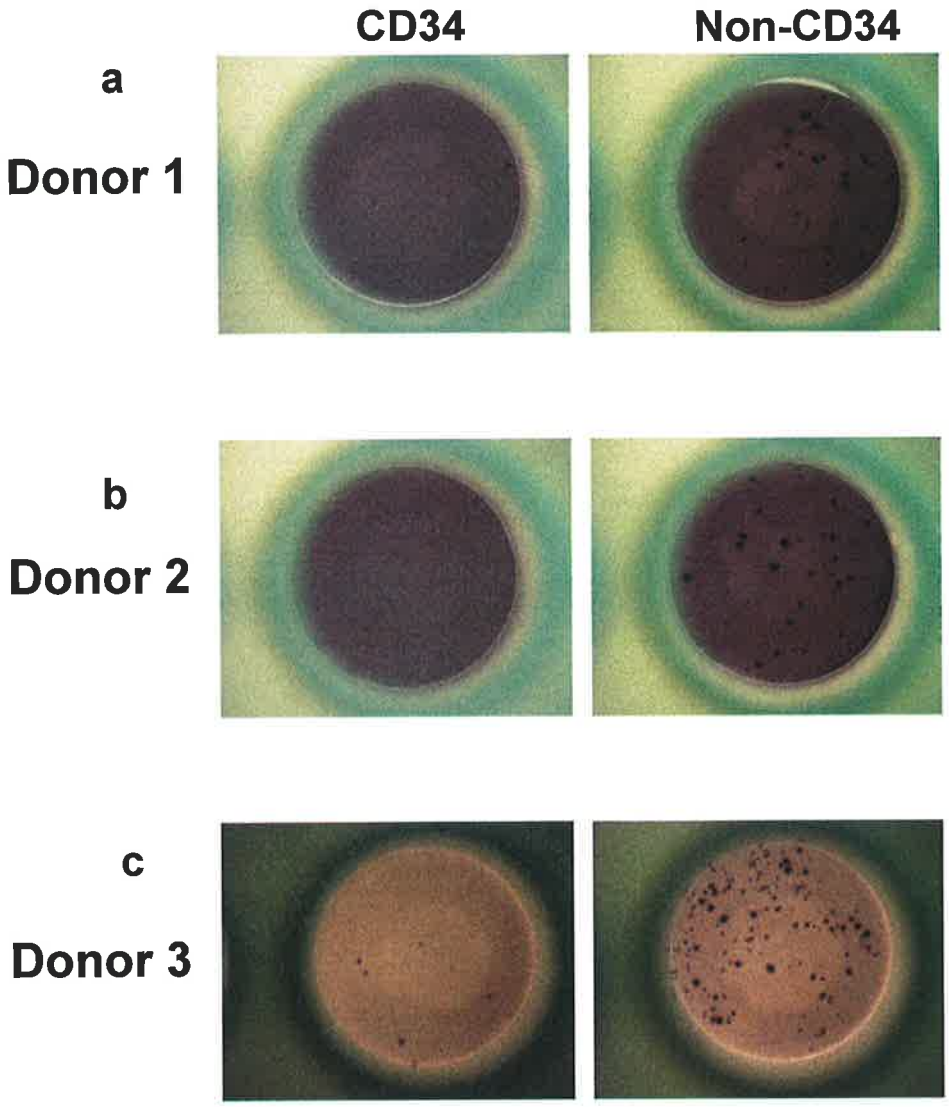


Figure 5.12. Normal donor cells

imatinib only (Figure 5.8, column a) strongly suggested that the predominant cytokines produced by non-CML CD34$^+$ cells was GM-CSF.

Interestingly, the number of non-CD34$^+$ CML cells in the mixed mononuclear cultures was actually less than the real number of CML CD34$^+$ cells, because non-CFSE labeled mixed mononuclear cells used in this experiment already contained CD34$^+$ cells at about 40-60%, whilst the CD34$^+$ only cultures contained nearly 100% CML CD34$^+$ cells. This suggested that the effect of bystander cells, but not CML CD34$^+$ cells was very strong to promote the survival and proliferation of CFSE-labeled CML CD34$^+$ cells and modulate the effect of imatinib (Figure 5.8 and 5.9, column b.).

Therefore, the cytokines produced by CML bystander cells were more important than potential production by CML CD34$^+$ cells because the level is high enough to enhance the effect of BCR/ABL compared with the threshold level of cytokines apparently produced by CML CD34$^+$ cells. This is quite surprising because previous reports focus on the effect of cytokine production by CML CD34$^+$ cells and did not examine the possibility of cytokine production by non-CD34$^+$ cells affecting cell survival and expansion of CML CD34$^+$ progenitors(Jiang, *et al* 1999).

The maintenance of CML bystander (non- CD34$^+$ cells) survival might be from the effect of autocrine cytokines produced by the bystander cells themselves or the effect of BCR/ABL. This is difficult to determine. Imatinib itself has little effect on mature CML cells. However, the normal granulocyte cells could survive in the circulation longer in the presence of GM-CSF as found in other study with the endothelium cells (Coxon, *et al* 1999). It might be possible that the autocrine cytokines from bystander themselves help to maintain cell survival rather than the effect of BCR/ABL, but it is more likely that low level of cytokines synergises with BCR/ABL activity.

The production of GM-CSF by non-CD34$^+$ cells was confirmed by ELISpot assay. This assay only detected the number of GM-CSF producing cells but not the level of GM-CSF. Therefore, the huge difference of number of black/blue spots may predict how much different the GM-CSF production was in different CML cultures, but is not in itself quantitation. This pattern of GM-CSF production by CML cells was similar to the normal

donor cells. Interestingly, it was also showed that imatinib could not impact on the production of GM-CSF by the bystander cells during short term cultures. Limitations of time and material prevented further characterisation of the cell type or type producing GM-CSF.

Therefore the role of bystander cells can be summarised 1) there were cytokines produced by bystander cells in this culture model, 2) the predominant cytokine was GM-CSF because the proliferation activity was blockable by E21R, the specific α receptor blocker, reducing to the base-line in the cells treated with imatinib alone in CML CD34$^+$ only cultures in Figure 5.8, and 3) other cytokines potentially produced by the bystander cell could not promote cell proliferation as well as GM-CSF did, but could maintain cell viability despite no proliferation because E21R could not pull the viability of cells in TMN cultures down to the same bottom-line as cells cultured with imatinib alone in CD34$^+$ only cultures (Figure 5.9).

Chapter 6.

Mechanism of protection from imatinib by GM-CSF

6.1. *Introduction*

6.1.1. *Overview from the previous results in Chapter 4 and 5*

The results on the responsiveness of CML $CD34^+$ CML cells to GM-CSF and bystander cells suggested that the BCR/ABL in primary CML cells did not fully activate all available signalling pathways, because added GM-CSF could enhance the spontaneous proliferation of these cells. Signalling pathways activated by BCR/ABL and GM-CSF receptors may be either unique or overlapping (Matulonis, *et al* 1993), therefore some pathways induced by GM-CSF may not be included in the activation downstream from BCR/ABL.

Many cell signalling pathways have been studied in BCR/ABL expressing cell lines. Imatinib has been used in those studies as an inhibitor to block the activity of BCR/ABL and examine the pathways involved in the activation through BCR/ABL. However, it is only relatively ABL specific and other kinases are blocked in a concentration-dependent manner. The activation of the Ras-Raf-Mek-Erk, Jak/STAT, PI3-K/Akt, c-Myc and also c-Jun signalling pathways in haematopoietic cells appears to be a direct consequence of BCR/ABL tyrosine kinase activity, playing a role in both cell proliferation and apoptosis. These pathways have been found to be involved after the activation of the GM-CSF-receptors as well. Interestingly, for signalling the cytokine receptors need a conformation change which occurs on binding their ligands. The binding between two receptor portions is required for the trans-phosphorylation of the β-common chain of the GM-CSF receptors.

6.1.2. *Role of normal c-ABL*

The function of c-ABL occurs mainly in the nucleus rather than in cytoplasm. This protein has various functions, including regulation of cell cycle checkpoints. Over-expression of c-ABL inhibits cell growth and leads to G1 arrest (Goga, *et al* 1995). The c-ABL was directly activated by various DNA damaging agents such as adriamycin. The ataxia-telangiectasia-mutated protein (ATM) also directly phosphorylates c-ABL in response to ionizing radiation, promoting p-53 dependent mechanisms of apoptosis and cell-cycle arrest (Baskaran, *et al* 1997). DNA-PK, a kinase involved in DNA repair, phosphorylates

c-ABL in response to stress, leading to its activation (Cibelli, *et al* 2002, Kharbanda, *et al* 1997). Recently, c-ABL has reportedly been shown to induce apoptosis through the up-regulation of EGR1 (early growth response-1 protein) expression (Stuart, *et al* 2005). EGR-1 expression is stimulated by cellular stress, such as DNA damage, hypoxia, and chemical treatment (Sakamoto, *et al* 1991, Thiel and Cibelli 2002). It was also known as a universal protein which has a quick-response region to a number of stimuli, such as stimulation with many environmental signals including growth hormones, factors, and neurotransmitters (Thiel and Cibelli 2002). The c-ABL activates the EGR-1 promoter by activating the mitogen/extracellular receptor kinase (MEK/ERK)-signaling pathway, leading to serum response elements (SREs) binding in the EGR1 promoter. The EGR-1/SREs complex binds the serum response transcription factor (SRF) and Elk1 transcription factor in response to growth factors (Shaw, *et al* 1989). Therefore, EGR-1 has roles in both survival and apoptotic pathways, involving the cytoplasmic activity of c-ABL. The ability of EGR-1 to associate with BCR/ABL has not been elucidated. The difference in the conformation between c-ABL and BCR/ABL may be the reason for any difference in the activation of EGR-1, explaining why c-ABL, once it is activated, predominantly involves cell cycle arrest, which is in contrast to BCR/ABL.

6.1.3. *Mechanism of c-ABL shuttling between nucleus and cytoplasm*

Recently, 14-3-3 was found to bind with phosphorylated c-ABL and inhibit c-ABL shuttling into the nucleus. The binding occurs at the nuclear import region on c-ABL protein after it is phosphorylated. The function of cytoplasmic c-ABL still involves cell apoptosis in response to stress rather than cell survival. No evidence of survival induction has been shown to involve the activation of c-ABL.

Progressive changes in the leukaemogenic signalling have been found in the BCR/ABL positive cells. BCR/ABL distorts the function of c-ABL. Although BCR/ABL involves in both survival and apoptotic signals, in contrast to c-ABL, it predominantly benefits cell survival. In BCR/ABL-positive cell lines, this protein seems to overcome the effect of activated cytokine receptors resulting in cell growth and resistance to apoptosis when deprived of cytokines (Bedi, *et al* 1994, McGahon, *et al* 1994, Sirard, *et al* 1994). Factors within the BCR/ABL may induce this protein to have predominant activation through the

survival pathways and inhibit apoptotic pathways. One is the nuclear import region in the ABL portion which is believed to be bound with 14-3-3 preventing BCR/ABL from entering the nucleus. Activated c-Jun N-terminal kinase induced by oxidative stress and DNA damaging agents is believed to phosphorylate 14-3-3 and lead to the separation of 14-3-3 from activated ABL(Yoshida, *et al* 2005), and also leads to the migration of ABL to nucleus (Yoshida, *et al* 2005). This occurs when using imatinib to inhibit BCR/ABL function (Vigneri and Wang 2001). This suggests that BCR/ABL is strongly involved in the inhibition of the DNA damaging and in turn to prevent the separation of 14-3-3 from BCR/ABL to prevent the nuclear migration of BCR/ABL.

6.1.4. *Control of cell apoptosis*

The activity of c-Jun is activated by growth factor deprivation as well. Therefore, BCR/ABL may inhibit the activation of c-Jun. The inhibition of c-Jun also affects other pro-apoptotic signals such as induction of Bim binding to LC8 and leading to inactivation of Bim binding to anti-apoptotic protein Bax and Bcl-XL. Recently, Bim has also been found to reduce its transcription in the presence of BCR/ABL and activated IL-3 receptors (Kuroda 2005).

Activation through STAT-5 and PI3-kinase/Akt pathway is also directly activated by BCR/ABL. Stimulation of Akt was reported to be dependent on the intact SH2 domain of BCR/ABL (Skorski, *et al* 1997). Recently, Akt was found activated in 32DcI3 cells expressing BCR-ABL /SH2-nil mutant after several weeks of *in vitro* culture (Nieborowska-Skorska, *et al* 1999). STAT-5, which is activated by the signalling from the complex of SH3 + SH2 domains of BCR/ABL (Nieborowska-Skorska, *et al* 1999), was simulated in several clones expressing BCR/ABL with SH3-nil /SH2-nil mutant cultured *in vitro* for several weeks. The re-activation of Akt and STAT-5 was suggested accompanied by increase of the resistance to apoptosis, induction of growth-factor independent proliferation and leukaemogenesis in SCID mice (Nieborowska-Skorska, *et al* 1999). Moreover, the study above also found that inhibition of Akt and/or STAT-5 activity by Akt or STAT-5 dominant negative mutants, respectively, inhibited the transformed phenotype of these cells, which again became sensitive to apoptosis induced by growth factor withdrawal and less leukaemogenic in mice (Nieborowska-Skorska, *et al* 1999). Therefore,

growth factor or cytokines could still not be excluded for this re-activation, because all cells were cultured constantly in the presence of cytokines (Nieborowska-Skorska, *et al* 1999). Interestingly, mutations re-activating Akt and STAT-5 occurred in cells expressing BCR/ABL mutants but not in parental 32Dcl3 cells (Nieborowska-Skorska, *et al* 1999).

6.1.5. *The regulation of apoptosis by Bim*

Shinjyo *et al.* in 2001 which showed that the phosphorylated Bim was induced by cytokine IL-3 (Shinjyo, *et al* 2001). Actually, in that study, if carefully examining the result, the phosphorylated Bim was also shown to be arisen from the apoptotic stimuli rather than IL-3 (Shinjyo, *et al* 2001). How Bim is phosphorylated by apoptotic stimuli is still unknown. The function of Bim has been reported so far was arisen from four mechanisms.

Firstly, the expression of Bim m-RNA which is down-regulated through the RAS/MAPK and PI3-K pathways, independently by the activation of cytokines was shown in mouse IL-3-dependent Baf-3, FL5.12, and 32D cells and the phosphorylation of Bim should be inhibited by this mechanism (Dijkers, *et al* 2000, Shinjyo, *et al* 2001). Furthermore, nerve growth-factor suppress Bim mRNA expression through the inactivation of the c-jun NH_2-terminal kinase in NGF-dependent neuron cells, in primary cultures of rat sympathetic neurons and in neuronally differentiated PC-12 cells (Biswas and Greene 2002) (Putcha, *et al* 2001, Whitfield, *et al* 2001). Finally, serum deprivation of CC139 fibroblasts up-regulate Bim mRNA through the classical MEK/extracellular signal-related kinase (ERK) pathway (Weston, *et al* 2003).

Secondly, subcellular localisation of Bim is controlled by IL-3 in FDC-P1 cells, another mouse IL-3 dependent lines, and by exposure to UV light in 293 cells ((Lei and Davis 2003, Puthalakath, *et al* 1999). Only Bim_{EL} and Bim_L form a complex with an M_r 8,000 dynein light chain, LC8 (also PIN or Dlc-1) (Crepieux, *et al* 1997, Jaffrey and Snyder 1996, King, *et al* 1996). IL-3 can induce the formation of the complex and IL-3 withdrawal released the complex from sequestration in the cytoplasm by mechanism not yet understood. However, in the case of 293 cells exposed to UV, phosphorylation at Thr-56 of human Bim_L by activated c-jun NH_2-terminal kinase appear to play an important role in this process (Lei and Davis 2003).

Thirdly, nerve growth factor has been found to phosphorylate Bim_{EL} and Bim_L but not Bim_S via the MEK/MAPK pathway in neuronal differentiated PC-12 cells (Yao and Cooper 1995). The phosphorylation of (rat) Bim_{EL} at Ser 109 and Thr 110 was reported to suppress the pro-apoptotic function of Bim_{EL} without affecting its binding potential to LC8 or its subscapular localisation (Biswas and Greene 2002). This suggested that the phosphorylation of Ser 109 and Thr 110 might help Bim to bind with LC8 faster and more tightly, because only with the binding of Bim to dynein light chain (LC8), of the microtubular motor complex, could be sequestered away from other Bcl-2 family members (Puthalakath, *et al* 1999). Pro-apoptotic function of Bim is arisen from the binding of Bim to Bcl-XL and BAX to inhibit the dimerization of these two proteins to cause anti-apoptosis and apoptosis respectively. This event occurrs in only Bim_{EL} and Bim_L, but not Bim_S.

Bim_{EL} and Bim_L were reportedly phosphorylated by IL-3 in Baf-3 cells using the same pathways that control the expression of Bim such as the Ras/Raf/MAPK and Ras/PI3-K pathways (Shinjyo, *et al* 2001). Although the BCR/ABL protein in BCR/ABL positive cell lines could inhibit Bim expression using the same pathways as IL-3 receptors, the Bim protein was still detected at a high level in the IL-3 receptor positive BCR/ABL cells cultured in IL-3 free medium rather than in IL-3 supplemented medium (Shinjyo, *et al* 2001). As higher Bim level was known to induce apoptosis, this suggested that these BCR/ABL cells could exhibit some of survival in medium without IL-3. In addition this finding also suggested that the phosphorylation of Bim by IL-3 or BCR/ABL may be not the important site for Bim to counteract with other anti-apoptotic proteins as has been questioned in the beginning. However, the phosphorylation of Bim may be important to inhibit Bim to bind with the anti-apoptotic protein and induce cell survival.

Fourth, proteasome-dependent degradation is involved in the regulation of Bim expression in serum-deprived fibroblasts and macrophage colony-stimulating factor-dependent osteoclasts (Akiyama, *et al* 2003).

Therefore, there is a lot of cross-reactivity of cytokine activity on Bim and maybe the function of Bim may need other apoptotic mechanisms to enhance its affect on cell

survival. This may be another mechanism for GM-CSF to cross-talk with BCR/ABL in control of cell apoptosis.

6.2. *Aims of this study*

An engineered cell line with both BCR/ABL and complete GM-CSF receptor (wt-BCR-ABL/CTLEN (as described in table 2.3) was chosen as a tool to investigate the interplays between the two signalling events and examine the downstream effect on cell proliferation and apoptotic control.

6.2.1. To determine if some signals were still not fully activated by BCR/ABL and were enhanced by GM-CSF.

6.2.3. To examine that although GM-CSF could not override the effect of BCR/ABL, it could enhance the signals involving apoptosis in cultures with imatinib.

6.2.4. To explain the mechanism by which GM-CSF allows wt-BCR-ABL/CTLEN to escape the inhibitory effect of imatinib.

6.3. *Special methods*

6.3.1. *MTS assay to evaluate the bio-kinetics of cell responses to imatinib and GM-CSF*

Wt-BCR-ABL/CTLEN cells containing complete GM-SF receptors were cultured in RPMI plus 10% FCS until the cells reached the exponential growth. Cells then were washed and resuspended in RPMI plus 0.5% FCS twice using centrifuge at 400xg for 10 minutes. Then cells were resuspended in serum deprived medium. Forty thousands of cells were seeded in 96-well plate in 100 μL SDM supplemented with imatinib, GM-CSF or E21R as indicated. The triplicated experiments were done in each condition to confirm the similarity. Figure 6.1 was shown the cell cultured in SDM supplemented with the titrated concentration of GM-CSF over the range 0-1x10^5 pg/mL and 3 μM imatinib and 10 μg/mL E21R. Figure 6.2 showed other cell lines were treated with imatinib over 0-10 μM and 300 pg/mL GM-CSF and 10 μg/mL E21R. Figure 6.3 showed wt-BCR-ABL/CTLEN cultured in SDM supplemented with imatinib over 0-100 μM and 1x10^4 pg/mL GM-CSF and 10

μg/mLE21R. Cells in all experiments were cultured for 3 days before adding MTS reagent at 20 μL/well and incubating at 37° C-incubator for 4 hours. The developing colour was read by ELISA plate reader set at 490 nm.

6.3.2. *Western immunoblot to study pathways involving apoptosis*

6.3.2.1. *Western immunoblot with total cell lysate*

The cell number of 5-10x 10^5 cells of the wt-BCR-ABL/CTLEN cells were seeded in 1 mL of starved medium containing 0.5% FCS/ RPMI supplemented with imatinib for 2 hours or otherwise as indicated. Then some sets of cell cultures had 10 nM GM-CSF added 10 minutes before the time for harvesting. In some setting, cells were treated with 20μM Etoposide and 40ng/mL PMA as the control for the Bim signalling. To stop cell activity 50 μL cold-PBS was added to the cell suspension after harvesting. The cultured-cells were was thrice in cold PBS. The cell pellet after washing was then lysed with 1% NP-40/ 1x TSE and incubated on ice for 30 minutes. The lysate was centrifuge at 16,000xg for 20 minutes to get rid of the cell fragment. The clear cell lysates were added with 4x loading buffer and boiled for 5 minutes before loading on a 10% SDS-PAGE gel.

6.3.2.2. *Western immunoblot with immunoprecipitate samples*

Cells were cultured at the density described in 6.3.2.1. The total cell number used per condition sample is 1x10^7 cells. After harvesting, the cell reaction was stopped using 50 μL cold PBS and cells then were washed thrice. The cell pellet was re-suspended in 1 mL 1% NP-40 / TSE lysis buffer and incubated on ice for 30 minutes. Then cell lysate was centrifuged at 16,000xg for 20 minutes to remove cell fragments. The lysate supernatant of 500 μg was transferred to the new eppendorf. Then 1 μg of specific antibody was added to the lysate and the cell lysate was incubated overnight with gentle rotation at 4 °C. On the next day, the lysate was added with 50 μL of Protein A Sepharose beads, and incubated at 4 °C with gentle rotation for 4 hours. The beads then were pelleted using centrifuge at 16,000 xg for 5 minutes at 4 °C. After discarding all the supernatant, the pellet were re-suspended with 30 μL of 1% NP-40 / TSE and mixed well. Then 10 μL of 4x loading

buffer was added to the lysate. The lysate, after adding loading buffer, was boiled 5 minutes before loading on a 10% SDS-PAGE gel.

6.4. Results

6.4.1. *Wt-BCR-ABL / CTLEN cells survived imatinib exposure in the presence of GM-CSF, which was blocked by the specific GM-CSF antagonist E21R.*

The MTS assay was used to evaluate the survival of wt-BCR/ABL transfected CTLEN cells. Briefly, forty thousands of wt BCR/ABL-CTLEN cells were seeded in SDM in 96-well plates. The cultures were supplemented with GM-CSF over the range 0 – $1X10^5$ pg/mL as the control in each plate (as shown in Figure 6.1, panel a. to c., the control line comes from the data of cells treated with the titrated GM-CSF only). Cells treated with GM-CSF plus 3 μM imatinib and/or 10 μg/mL E21R were examined to compare their survival with the control cells. Triplicate experiments were performed for each condition as shown in Figure 6.1, panels a. to c. The individual panels were derived from an individual plate. In all panels, the cells cultured with SDM plus GM-CSF only were used a the control.

After 3 days of culture, wt-CTLEN/BCR-ABL cells grew well without GM-CSF as shown in all panels in Figure 6.1., and GM-CSF could not enhance growth and/or survival of this cell line. Panel a showed that imatinib completely inhibited cell growth compared with cells without treatment. GM-CSF was able to override imatinib when its concentration was greater than 1000 pg/mL and could provide equal survival to controls when its concentration reached 10 ng/mL (or 1 x 10^4 pg/mL). Panel b, E21R used was not able to suppress the cell survival compared with no treatment. At every point of GM-CSF concentration, cells had the same MTS value compared with the control (treated with GM-CSF only). To confirm that 10 μg/mL E21R was sufficient to suppress the effect of GM-CSF up to the concentration of 100 ng/mL (or 1 X 10^5 pg/mL), E21R was added to the cultures with GM-CSF and imatinib. E21R could suppress cell survival down to nearly the same level as the cells treated with imatinib only, as shown in panel c compared with panel a.

Figure 6.1. Wt-BCR-ABL/CTLEN cell proliferation was inhibited by imatinib

Wt-CTLEN/BCR-ABL cells were cultured in 96-well plates for 3 days with the GM-CSF over the range 0-10^5 pg/mL in the presence or absence of 3 μM imatinib. Then the cultures were assessed for the cell proliferation activity by MTS assays. The controls were cells with GM-CSF alone.

Panel a., imatinib was very effective to inhibit cell proliferation and reduce the OD-value down to about 0.05 over the background. To reach the maximal activity of BCR/ABL (about 0.8), at least 10 ng/mL (or 10,000 pg/mL) was needed. This is the level of GM-CSF which was planned to use in Western Blot experiment.

Panel b., E21R only was added to inhibit the binding of GM-CSF to its receptors. E21R was not found to reduce the cell survival compared with the reference line data. This suggested that the wt-CTLEN/BCR-ABL could survive without added GM-CSF.

Panel c., E21R added to the cells treated with imatinib and titrated-GM-CSF has shown that it could not completely reduce cell survival down to ~0.05 as seen in panel a. The OD-value when adding E21R to the cell treated with imatinib+GM-CSF was around 0.2 and quite similar at every point of the GM-CSF level. This suggested E21R at 10 pg/mL could definitely block the activity of GM-CSF receptors. In addition, E21R itself might partially stimulate the signals through GM-CSF receptors, which allowed cells to survive imatinib and increased cell survival up to 0.2 over the blackground compared with the cells treated with imatinib+GM-CSF in panel a.

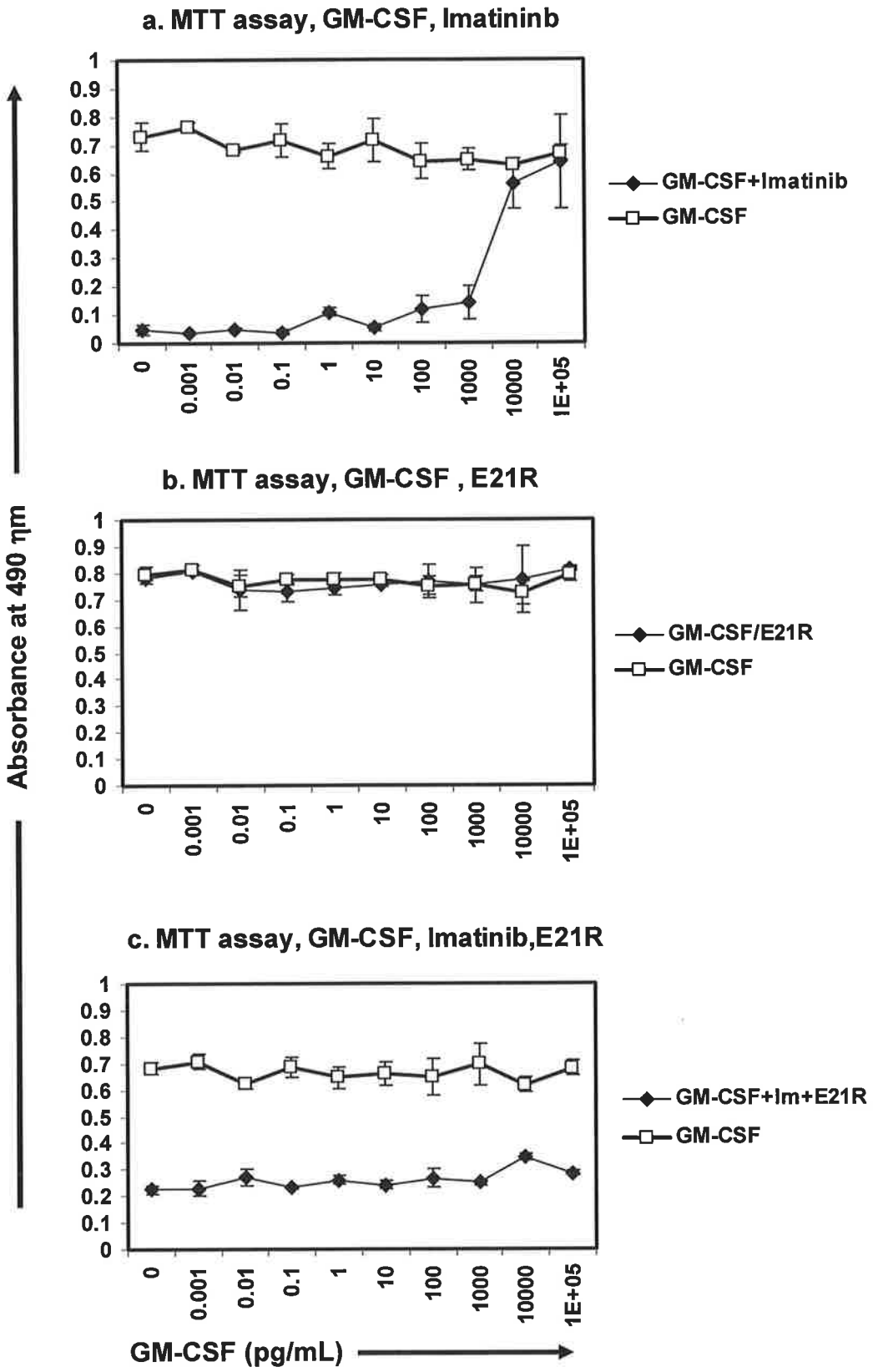

a. MTT assay, GM-CSF, Imatininb
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.001
0.01
0.1
1
10
100
1000
10000
1E+05
GM-CSF+Imatinib
GM-CSF
b. MTT assay, GM-CSF , E21R
GM-CSF/E21R
GM-CSF
Absorbance at 490 ηm
c. MTT assay, GM-CSF, Imatinib,E21R
GM-CSF+Im+E21R
GM-CSF
GM-CSF (pg/mL)

Figure 6.1

6.4.2. *GM-CSF could clearly enhance and maintain the survival of wt-BCR-ABL/CTLEN in the presence of imatinib compared with other BCR/ABL expressing cell lines.*

A number of BCR/ABL cell lines were used to examine their responses to GM-CSF and escape from imatinib inhibition, including K562, KU812, Meg-O1, MOLM-1, including the engineered line - wt-BCR-ABL/CTLEN. All were cultured in SDM in 96-well plates supplemented with imatinib at 0 – 10 μM +/- 10 μg/mL E21R +/- 300 pg/mL GM-CSF. K562, KU812, MOLM-1 and MegO1 showed sensitive responses to imatinib, but only KU812 showed enhanced survival in the presence of imatinib when 10 ng/mL GM-CSF was added. However, the effect of GM-CSF on KU 812 could only induce a little shift of OD value over imatinib treatment. Although this shift is significant compared with the cell without treatment, it did not reach the level of spontaneous proliferation.

The engineered cell lines CTLEN containing complete human GM-CSF receptor and the further engineered line wt-BCR-ABL/CTLEN, which is the CTLEN line containing human BCR/ABL, were used to test the responses of cells on imatinib in the presence or absence of GM-CSF (Figure 6.2 panel e and f). The parental CTLEN cells without GM-CSF show low cell survival by MTS assay, but at high concentration of imatinib the survival of cells was decreased. However, added GM-CSF markedly induced cell survival. In contrast to the CTLEN line, GM-CSF could not affect the spontaneous cell survival of the wt-BCR-ABL / CTLEN. However, GM-CSF could maintain the cell survival nearly to similar levels as their spontaneous survival, in the presence of high concentration of imatinib. Therefore, the wt-BCR-ABL/ CTLEN line might be useful to investigate the response of cells to imatinib and GM-CSF by Western Blot protein assay of the key signalling molecules.

6.4.3. *GM-CSF showed to maintain the survival of the wt-BCR-ABL / CTLEN even at high concentration of imatinib, this effect was blocked by E21R.*

After the optimal concentration of GM-CSF used to maintain the survival of wt-CTLEN/BCR-ABL cells was determined, as shown in Figure 6.3, 10 ng/mL of GM-CSF was used to examine if this cell could maintain cell survival when imatinib was titrated

Figure 6.2. BCR/ABL cell lines were examined their growth in cultures with imatinib and GM-CSF.

BCR/ABL positive cell lines in the figure included Meg-01 - the b2a2 positive megakaryoblast cell lines, MOLM-1 - the b2a2 fusion myeloid cells, KU812 - the b3a2 fusion myeloid cells, K562 - the b3a2 fusion erythroblast cells, the Wt-BCR-ABL/CTLEN - the mouse cytotoxic T-cells with human b3a2/ BCR-ABL and complete human a-specific and b-common receptors of GM-CSF, and CTLEN cells - the mouse cytotoxic T-cells with human α-specific and β-common receptors of GM-CSF.

Imatinib is very effective to inhibit cell proliferation of most of the cell lines except the CTLEN which has no BCR/ABL. The response to GM-CSF of the cell lines in panels a-d was not observed except KU812 (panel c) which has only marginal response. CTLEN cell line without BCR/ABL cells were GM-CSF dependent, therefore it respond to GM-CSF very well. In CTLEN with BCR/ABL in panel f, GM-CSF could not not enhance cell proliferation over the spontaneous proliferation driven by BCR/ABL. When the concentration of imatinib is sufficient to inhibit BCR/ABL function, GM-CSF was found to maintain the proliferation of the wt-BCR-ABL/CTLEN overriding the effect of imatinib.

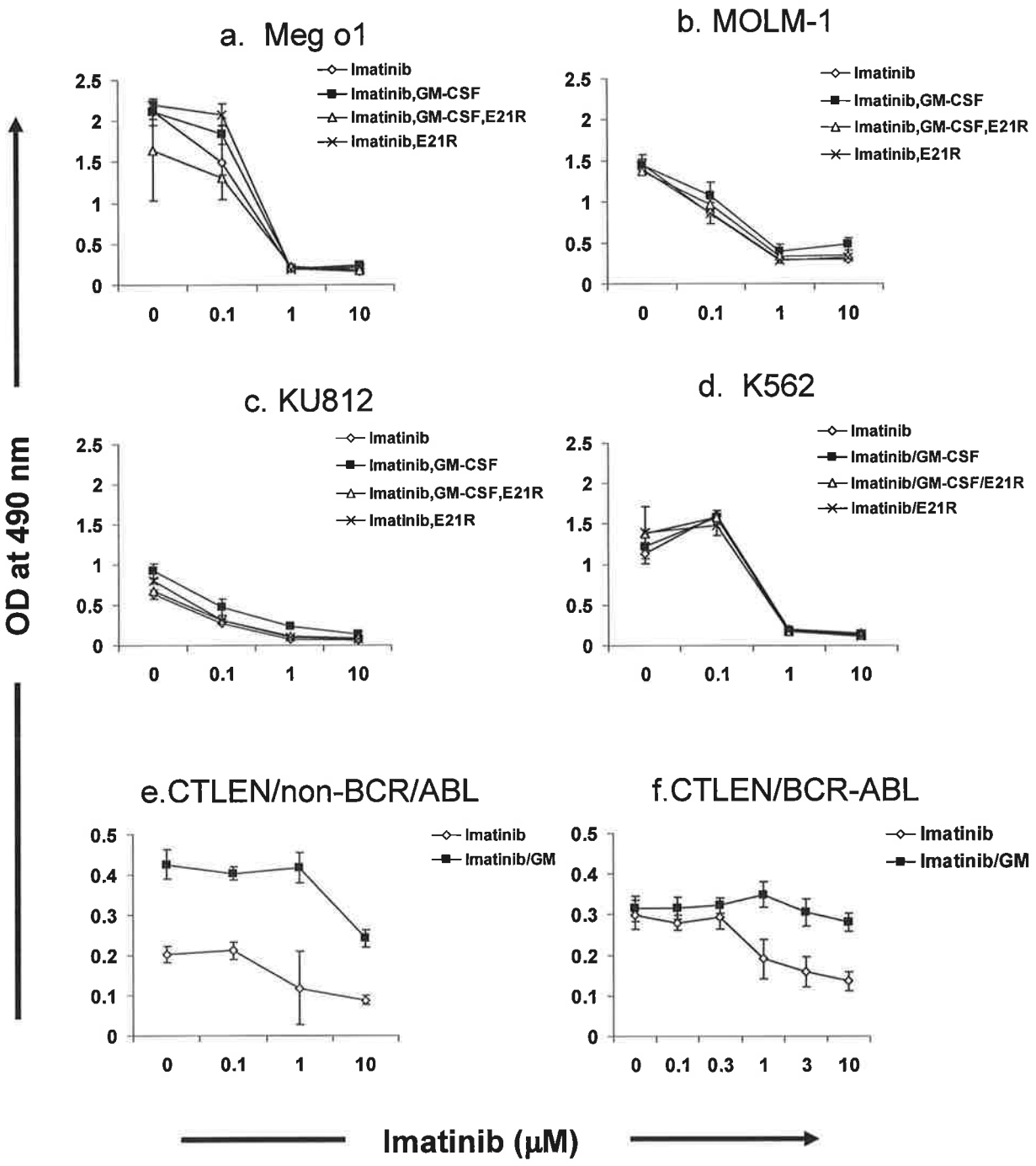

a. Meg o1
Imatinib
Imatinib,GM-CSF
Imatinib,GM-CSF,E21R
Imatinib,E21R
b. MOLM-1
c. KU812
d. K562
Imatinib/GM-CSF
Imatinib/GM-CSF/E21R
Imatinib/E21R
e.CTLEN/non-BCR/ABL
Imatinib/GM
f.CTLEN/BCR-ABL
OD at 490 nm
Imatinib (μM)

Figure 6.2

Figure 6.3. Ten ng/mL of GM-CSF was able to promote the survival of wt-CTLEN/BCR-ABL in the presence of imatinib.

For these experiments, the concentration of GM-CSF and E21R were fixed at 10 ng/mL and 10 μg/mL respectively. Imattinib was titrated over the range 0 to 100 μM in order to examine the maximal effect of imatinib on this type of cell line. The cells treated with imatinib alone were used as controls

Panel a, ten micro-gram/mL of E21R added to the cells treated with imatinib showed a partially promotion of cell survival and delay the concentration of imatinib to reach the maximal suppression of cell survival to ~ 30-50 μM.

Panel b showed that GM-CSF could maintain cell survival even at relatively high concentrations of imatinib compared with the controls.

The ability of GM-CSF to maintain viability in the presence of imatinib was confirmed by adding E21R in panel c.

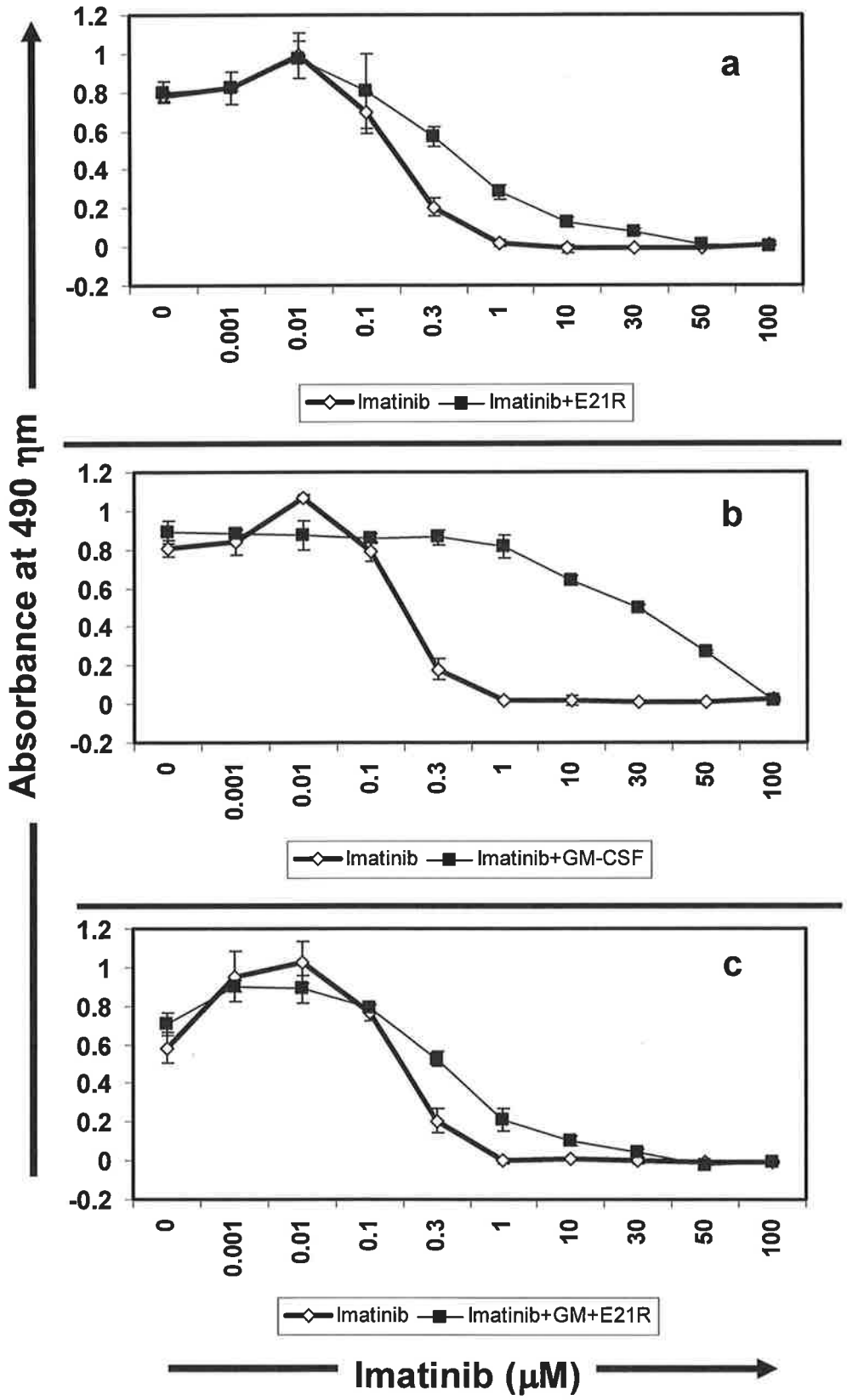

Absorbance at 490 ηm
a
b
c
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.001
0.01
0.1
0.3
1
10
30
50
100
Imatinib
Imatinib+E21R
Imatinib+GM-CSF
Imatinib+GM+E21R
Imatinib (μM)

Figure 6.3

over the ranges 0 – 100 μM with SDM. Forty thousand wt-CTLEN/BCR-ABL cells were seeded in cultures for 3 days. E21R at 10 μg/mL was added to two 96-well plates to confirm that the GM-CSF effect was blockable. Imatinib was able to completely suppress cell survival at the concentration of 1 μM. The IC50 of imatinib inhibition of cell survival was about 0.3 μM (panel a). Added GM-CSF helped cells to resist imatinib by raising the IC50 to greater than 10 μM which is a generally toxic concentration of imatinib. (panel b.). E21R could block the effect of GM-CSF and reduce the IC50 of imatinib down to the same level of (about 0.3 μM) (panel c.). The pattern of the modulation of GM-CSF on the this cell line in response to imatinib is quite similar to the study of primary CML $CD34^+$ cells using dye dilution method in Chapter 3.

6.4.4. *Mechanism of imatinib-induced reacquisition of cytokine dependency in the wt-CTLEN/BCR-ABL cell line.*

6.4.4.1. *Imatinib did not change the level of p-Akt and GM-CSF could enhance the level of p-Akt in the presence of imatinib.*

To clarify the molecular mechanism involved in imatinib-induced reacquisition of cytokine dependecy, Western Blot analysis was used to show that some signals could be stimulated by GM-CSF after the function of BCR/ABL protein was inhibited using imatinib. This helped to confirm the data from the MTS assay. The Akt activity was investigated as it is the common signal through which apoptotic pathways are directly activated by activated GM-CSF common β receptor and BCR/ABL kinase activity. Imatinib could not suppress the phosphorylation of this protein and also with the titration of imatinib over the ranges 0-10 μM, the phosphorylated-Akt was reduced after a 2 hour-incubation. Imatinib at high concentration may reduce the phosphorylation of this protein due to the toxicity of imatinib to the cells in general. This is confirmed by taking the ratio between p-AKT and Akt which was increased(Figure 6.4) at higher level of imatinib. In addition, The increased level of p-Akt and p-Akt:Akt ratio in the presence of GM-CSF was also observed(Figure 6.4).

Using mouse-monoclonal anti-human Akt IgG1 to pull down all Akt in order to control the level of total Akt loaded in gel, the level of p-Akt appeared to have no change according to

Figure 6.4. Phospho-Akt evaluated to in CTLEN/BCR-ABL showed that it was decreased with the increased concentration of imatinib.

Although the level of p-AKT was decreased, the Akt loading in gel was decreased as well suggesting that higher concentration of imatinib may inhibit the production of some proteins. The decreased Akt has been confirmed by using b-actin detection of the protein loading. However, the proportion of p-Akt to Akt was increased when imatinib concentration was increased. Addition of GM-CSF to the cultures could enhance this ratio.

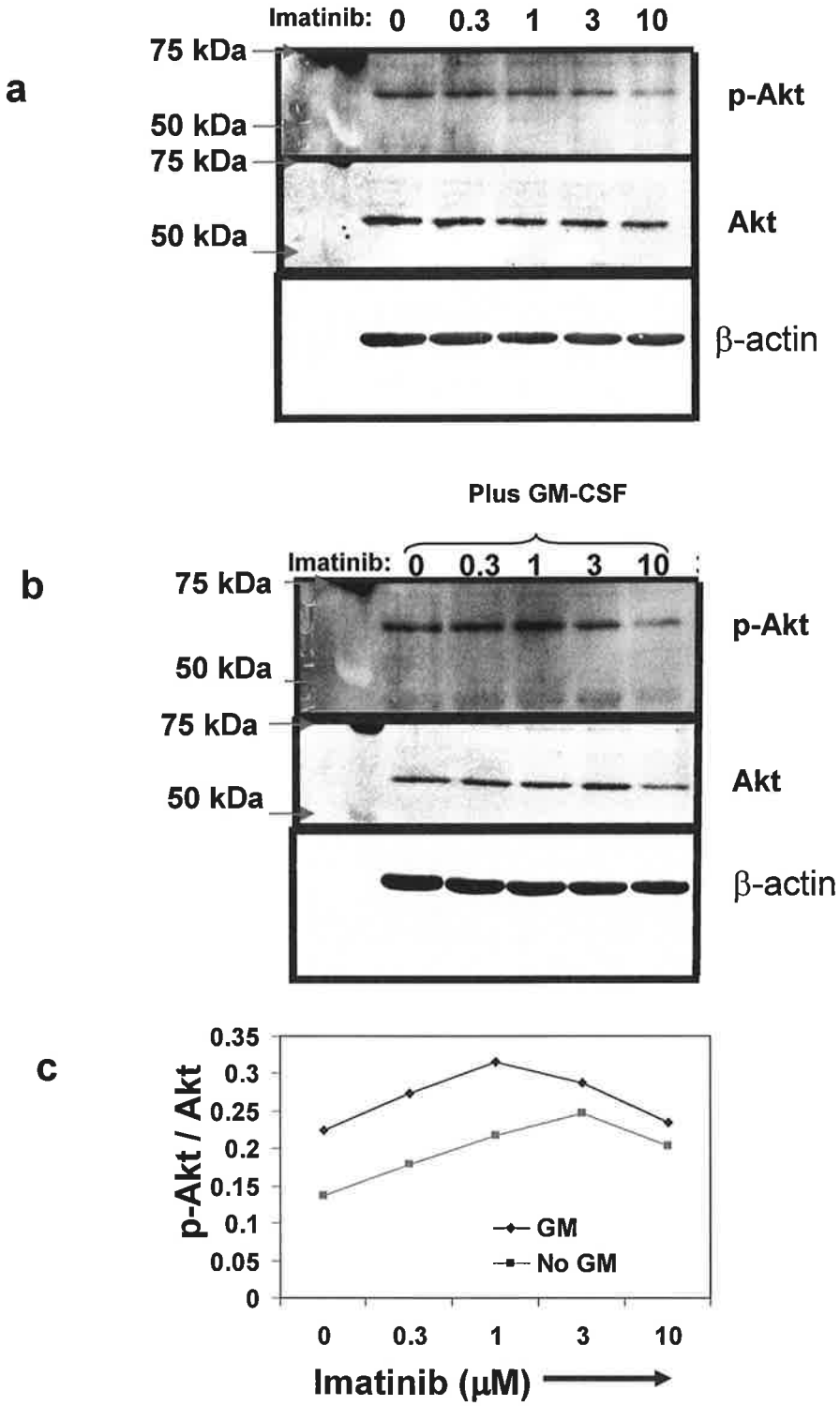

a
Imatinib: 0 0.3 1 3 10
75 kDa
50 kDa
75 kDa
50 kDa
p-Akt
Akt
β-actin
b
Plus GM-CSF
Imatinib: 0 0.3 1 3 10
75 kDa
50 kDa
75 kDa
50 kDa
p-Akt
Akt
β-actin
c
p-Akt / Akt
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
GM
No GM
0 0.3 1 3 10
Imatinib (μM)

Figure 6.4

the level of imatinib in cultures. In addition, added GM-CSF also enhanced the densitometry of p-Akt expression, but p-Akt level appeared equal at either points of imatinib (Figure 6.5). These data suggested that imatinib had no effect on the expression of p-Akt.

The p-Akt was shown to be increased in GM-CSF treated cultures compared with non-GM-CSF cultures, correlating with the increased 14-3-3 binding (Figure 6.5). Etoposide treated positive control cultures showed no change in the densitometry of p-Akt and also 14-3-3 binding activity in both gel. Considering the cells treated with imatinib plus PMA as the same control in both gel for cross-comparison, it was found that

1) GM-CSF induce p-Akt level in imatinib + GM-CSF higher than in imatinib only cultures;
2) The 14-3-3 level on the gels for cells treated with imatinib only and with imatinib + GM-CSF was similar. However, due to the level of p-Akt in GM-CSF added cultures being higher than imatinib treated cultures, this made the 14-3-3 binding activity to p-Akt be decreased in cultures with added GM-CSF;
3) PMA did not change the level of p-Akt compared with the imatinib-only cultures.
4) Etoposide could lower the level of p-Akt and again PMA did not change the level of p-Akt in cultures with Etoposide.

6.4.4.2. *GM-CSF also modulated Bim, a pro-apoptotic signal in the presence of imatinib.*

Bim is another protein which may be suppressed by BCR/ABL and the activated cytokine receptors such as by IL-3 and GM-CSF. Phosphorylated Bim_{EL} is involved in the inhibition of anti-apoptotic signals of the BCl-2 family. The densitometry of Bim_{EL} was increased when the concentration of imatinib was increased, particularly at the concentration greater than 1μM. The Bim_{EL} together with the other isotypes of Bim_{EL} was also found to be increased in cultures with GM-CSF plus imatinib (Figure 6.6). The isotypes of Bim, which have been shown by the slow migrating bands between 24-30 kDa, was increased in imatinib plus GM-CSF (panel b) but less in imatinib only (panel a.). However, the level of Bim_{EL} was increased overall in cultured with GM-CSF related to imatinib concentration.

Figure 6.5. Immunoprecipitation of Akt allowed the proportion of p-Akt and 14-3-3 binding to be deyermined.

All Akt protein was pulled down in cell lysate, derived from the culture of wt-BCR-ABL/CTLEN cells under condition as indicated, using mouse monoclonal Akt antibody and protein A sepharose beads. Then the immunoprecipitated lysate was resolved in 10% SDS-PAGE gel. The lysate of cells treated with imatinib plus PMA, Etoposide and Etoposide plus PMA was used as the control for the background variation.

PMA could not enhance the expression of p-Akt compared with imatinib only cultures, but not with imatinib plus GM-CSF suggesting the level of p-Akt with GM-CSF is little enhanced more than the level of p-Akt with imatinib only (panel b and a respectively). This was confirmed by re-probing with anti-Akt antibody and ratioing between p-Akt/Akt, which showed inceased when GM-CSF was added to cultures.

Panel e and f showed little decrease of 14-3-3 binding to Akt after treatment with imatinib. This binding could not be enhanced with GM-CSF, when comparing with the cells treated with imatinib plus PMA.

Panel g showed the ratio of corrected densitometry between p-Akt and Akt which was increased by added GM-CSF.

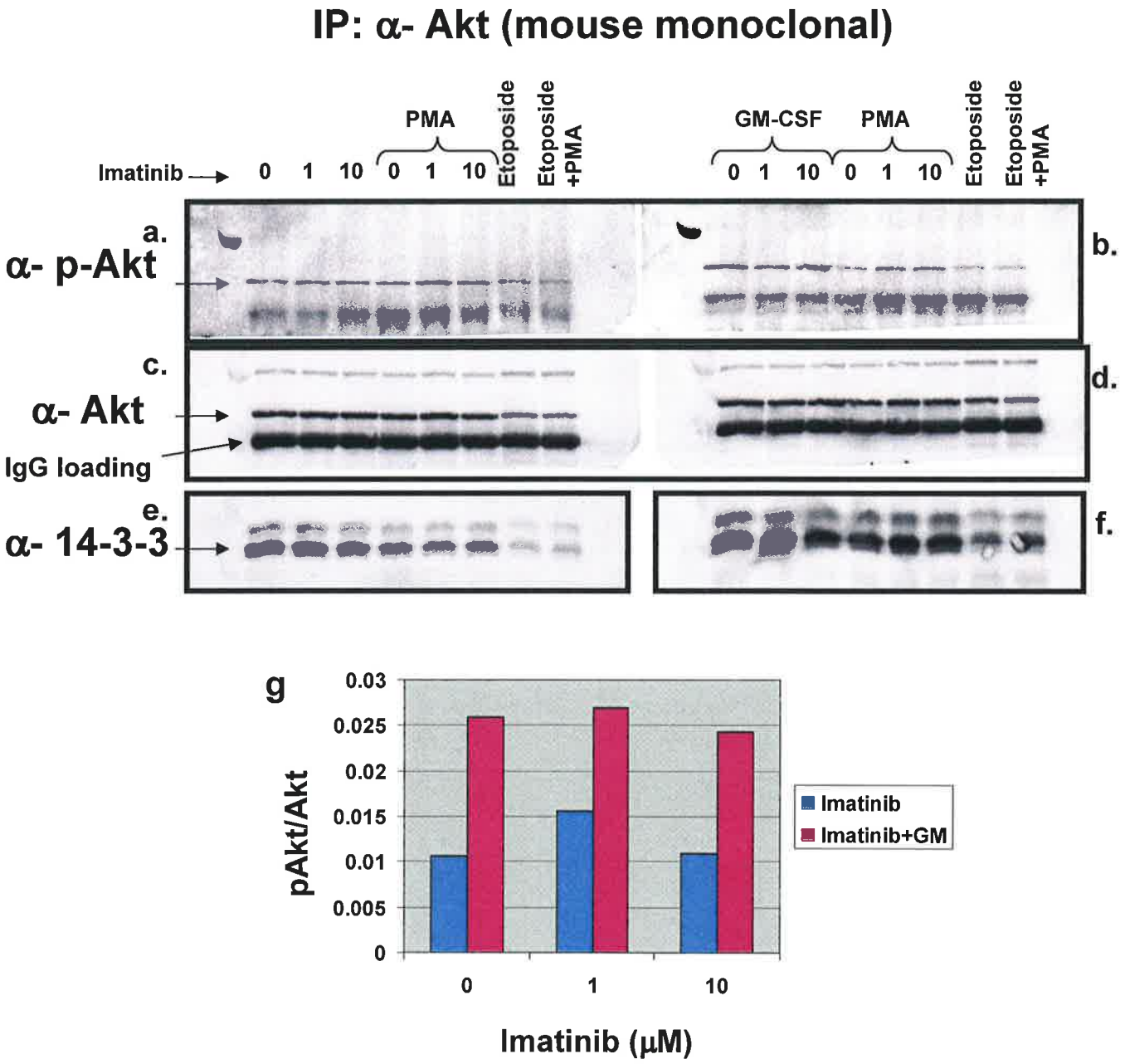

IP: α- Akt (mouse monoclonal)
Imatinib
0 1 10
PMA
0 1 10
Etoposide
Etoposide +PMA
GM-CSF
PMA
0 1 10 0 1 10
Etoposide
Etoposide +PMA
a.
α- p-Akt
b.
c.
α- Akt
IgG loading
d.
e.
α- 14-3-3
f.
g
pAkt/Akt
0.03
0.025
0.02
0.015
0.01
0.005
0
0 1 10
Imatinib (μM)
Imatinib
Imatinib+GM

Figure 6.5

Figure 6.6. Imatinib was shown to increase the level of Bim a protein associated with induction of apoptosis, which was reversed by GM-CSF.

Panel a. showed wt-BCR/ABL/CTLEN cells treated with imatinib over the range 0-5 μM, and showed the middle isoform of Bim_{EL} increasing when the concentration of imatinib was increased. This is associated with the increased production of Bim_{EL} and presumably also the decreased degradation in proteosomes. The increased production is important for induction of cell apoptosis.

Panel b showed overall levels of $Bim_{EL}$are decreased with added GM-CSF. However, the middle isoform became increased when the concentration of imatinib was increased, presumably because the production of Bim was decreased in the presence of GM-CSF, however it is likely the degradation also became decreased by the effect of high doses of imatinib.

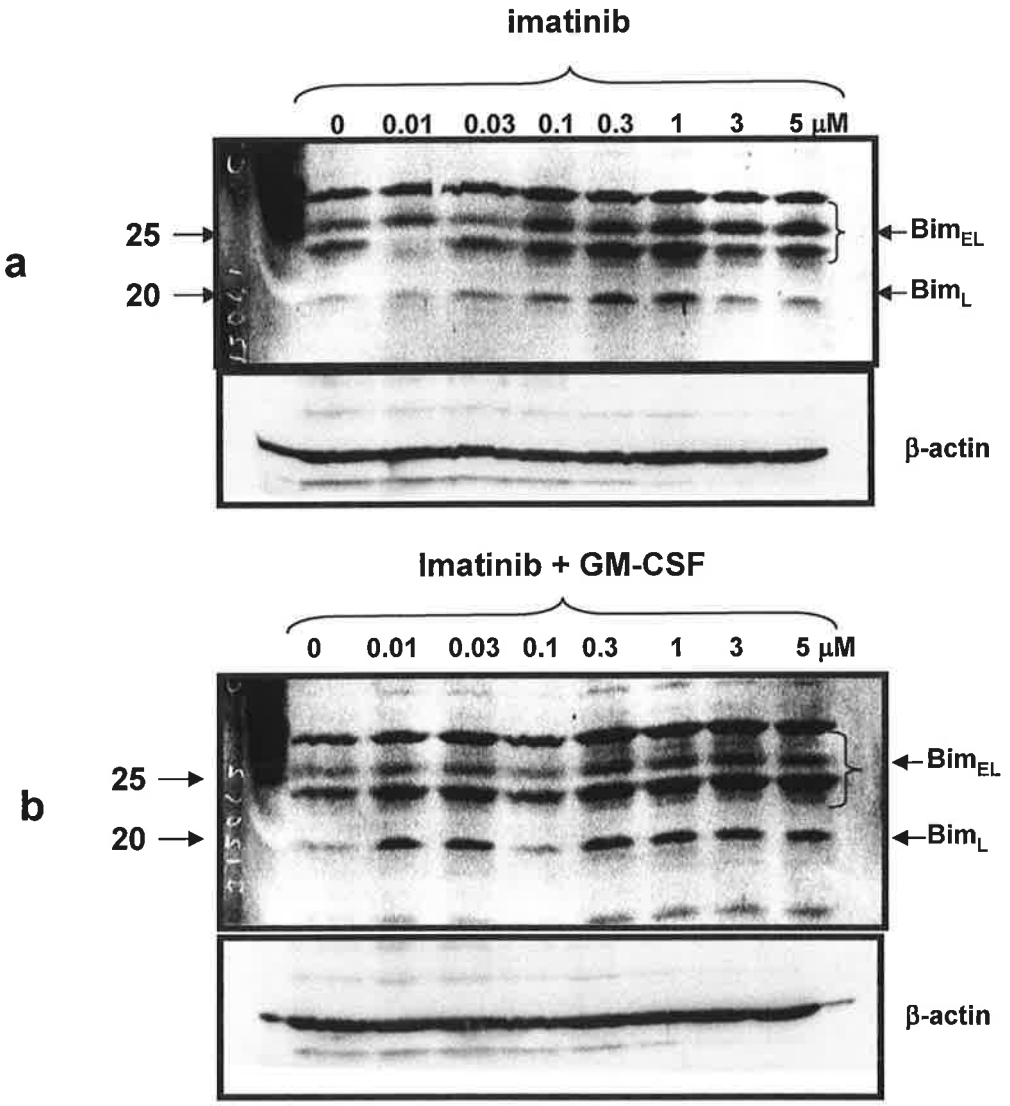

a
imatinib
0 0.01 0.03 0.1 0.3 1 3 5 μM
25
20
Bim$_{EL}$
Bim$_L$
β-actin
b
Imatinib + GM-CSF
0 0.01 0.03 0.1 0.3 1 3 5 μM
25
20
Bim$_{EL}$
Bim$_L$
β-actin

Figure 6.6

6.4.4.3. *Crkl was the predominant protein phosphorylated by BCR/ABL and was not phosphorylated by addition of GM-CSF when BCR/ABL was blocked by imatinib.*

To confirm that the wt-BCR-ABL / CTLEN cells responded very well to imatinib, imatinib was titrated over the range 0-30 μM (Figure 6.7). Imatinib could almost completely inhibit the phosphorylation of Crkl at imatinib levels greater than 1 μM. The calculated IC50 was around 0.5 μM. GM-CSF was found to marginally increase phosphorylated Crkl at 0.3 and 1μM imatinib but the IC50 to imatinib remained similar (around 0.7 μM). This cell line therefore was quite sensitive to imatinib. Although GM-CSF could marginally stimulate the active Crkl, the level of active Crkl was not increased to reach the non-treated control level. Therefore the survival of cells which was shown by MTS assay in figure 6.1, panel a. and 6.3, panel b. is likely to result from the activation of GM-CSF receptors on other signals rather than Crkl.

6.4.4.4. *Erk is constitutively phosphorylated at low level in the wt-BCR-ABL/CTLEN cell and phosphorylation is enhanced by GM-CSF..*

Erk was examined to evaluate this protein substrate relating to proliferation and apoptosis. The densitometry of non-active form Erk measured by Western blot was quite high but its phosphorylation in this cell line was not very strong. However, the thin phosphorylated bands were found decreasing as imatinib concentration increased (Figure 6.8, Panel a.), similar to that seen in phosphorylated-Crkl. GM-CSF could markedly enhance the amount of phospho-Erk and the level increases even in cultures with a high-concentration of imatinib (Figure 6.8, panel b).

6.4.4.5. *P-Akt (Ser473) is shown to bind with other proteins suggesting it interacts with those proteins resulting in phosphorylation.*

Total lysate of the cultured wt-BCR-ABL/CTLEN cells was immunoprecipitated with mouse monoclonal antibody to Akt, and protein-A sepharose. Then the immunoprecipitated lysate was resolved in 10% SDS-PAGE gel and transferred to PVDF. Then the membrane was probed by the specific antibody as indicated (Figure 6.9).

Figure 6.7. The wt BCR-ABL/CTLEN were treated with imatinib to evaluate the efficacy of imatinib in the inhibition of BCR/ABL in cultures with or without GM-CSF.

The wt-BCR-ABL / CTLEN cells were treated with imatinib over the range 0-30 μM for 2 hours in the 37 °C-incubator. Then 10 minutes before harvesting to obtain cell lysate, 10 ng/mL GM-CSF was added to the cultures. The imatinib IC50 of the inhibition of phosphorylated Crkl expression was compared between cells cultures with and without GM-CSF. The IC50 was calculated by the ratio of phosphorylation band of Crkl to the total bands of Crkl in terms of densitometry. GM-CSF does not alter the apparent IC50 of imatinib inhibition of BCR/ABL using p-Crkl expression as a surrogate of ABL kinase activity

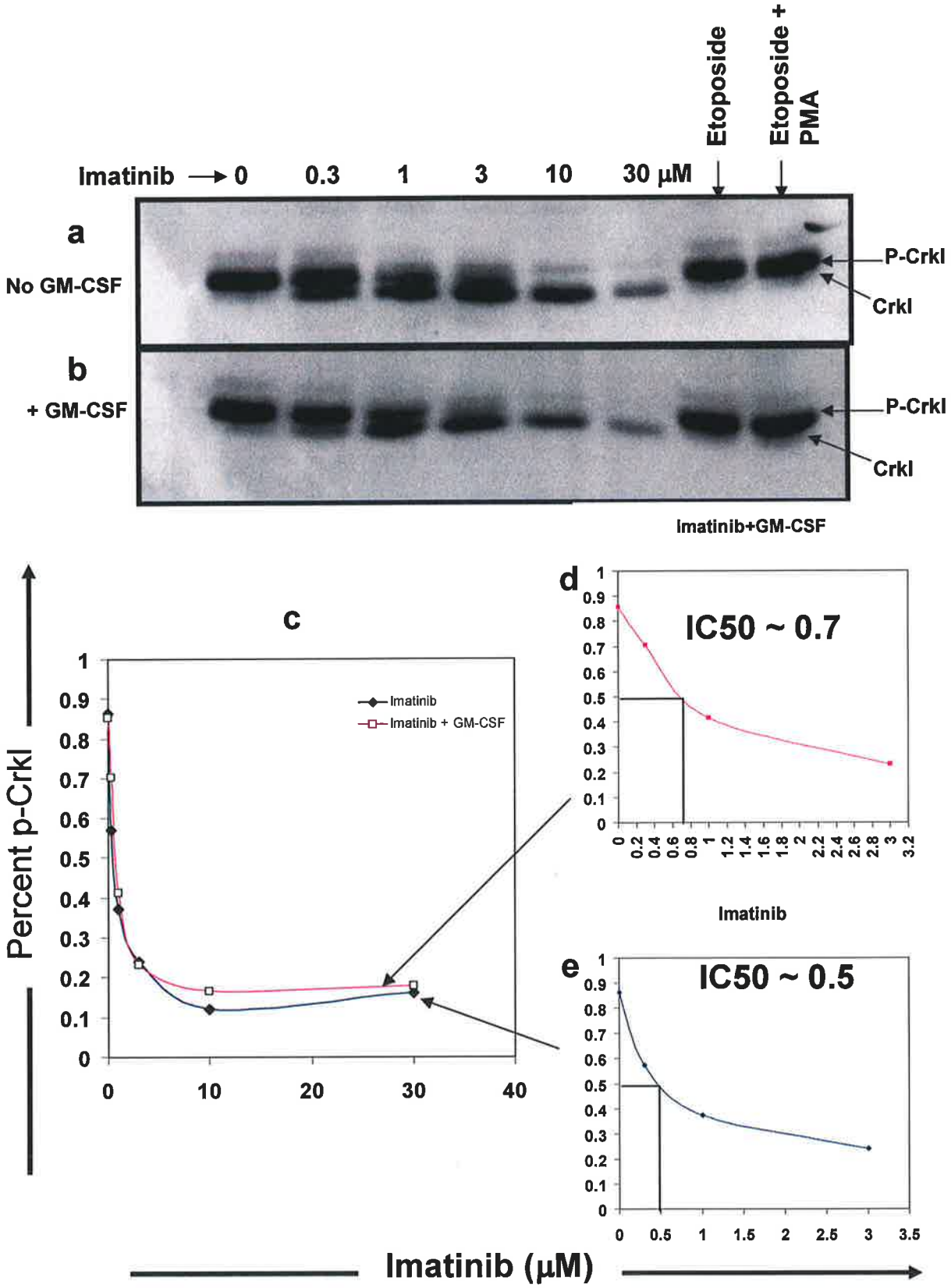

Etoposide
Etoposide + PMA
Imatinib → 0 0.3 1 3 10 30 μM
a
No GM-CSF
P-Crkl
Crkl
b
+ GM-CSF
P-Crkl
Crkl
Imatinib+GM-CSF
c
Imatinib
Imatinib + GM-CSF
Percent p-Crkl
d
IC50 ~ 0.7
Imatinib
e
IC50 ~ 0.5
Imatinib (μM)

Figure 6.7

Table 6.1. Densitometry on Western Blot for Crkl study

Imatinib treated CTLEN/BCR-ABL cells					
Imatinib (μM)	p-Crkl 1	BG	Corrected	Corr p+C	Ratio
0	174911.28	16717.23	158194.05	183593.68	0.861653026
0.3	136229.81	22624.03	113605.78	199446.11	0.569606396
1	94963.29	23671.15	71292.14	191478.64	0.372324245
3	73753.51	22352.84	51400.67	214085.53	0.240094088
10	34137.38	19314.15	14823.23	124077.35	0.119467655
30	24799.78	17199.36	7600.42	47616.39	0.159617728
Imatinib (μM)	Crkl 1	BG	Corrected		
0	42403.01	17003.38	25399.63		
0.3	107214.38	21374.05	85840.33		
1	142642.37	22455.87	120186.5		
3	181902.33	19217.47	162684.86		
10	127101.32	17847.2	109254.12		
30	55889.7	15873.73	40015.97		
Imatinib plus GM-CSF					
Imatinib (μM)	p-Crkl 2	BG	Corrected	Corr p+C	Ratio
0	208204.09	25317.8	182886.29	214420.98	0.852930949
0.3	137170.15	24793.38	112376.77	160019.06	0.702271154
1	96168.81	26042.08	70126.73	170335.13	0.411698573
3	55121.34	25250.61	29870.73	129646.71	0.230400987
10	37100.19	24555.34	12544.85	75946.41	0.16518029
30	29448.06	23406.73	6041.33	33790.79	0.178786291
Imatinib (μM)	Crkl 2	BG	Corrected		
0	55358.51	23823.82	31534.69		
0.3	71601.84	23959.55	47642.29		
1	124737.62	24529.22	100208.4		
3	123666.27	23890.29	99775.98		
10	86764.06	23362.5	63401.56		
30	50552.5	22803.04	27749.46		

p-Crkl = phosphorylated Crkl, Crkl 2 and p-Crkl 2 = Crkl and p-Crkl expression in cultures with GM-CSF, BG = background densitometry, corrected = densitometry of Crkl or p-Crkl minus background, Corr p+C = corrected p-Crkl plus corrected Crkl, Ratio = corrected p-Crkl / corrected p+C

Figure 6.8. GM-CSF could enhance the activity of Erk1/2 in wt BCR-ABL/CTLEN cells in the presence of imatinib

The wt-BCR-ABL / CTLEN cells were cultured in the presence of imatinib over the range 0 – 5 μM only (panel a) and with imatinib plus 10 ng/mL GM-CSF (panel b)

The p-Erk was not very high in the non-treated cells. However, its level became decreased with titrated imatinib in a dose-dependent manner. Added GM-CSF in the cultures with imatinib was able to enhance the level of its phosphorylated form and became increased when the concentration of imatinib was increased.

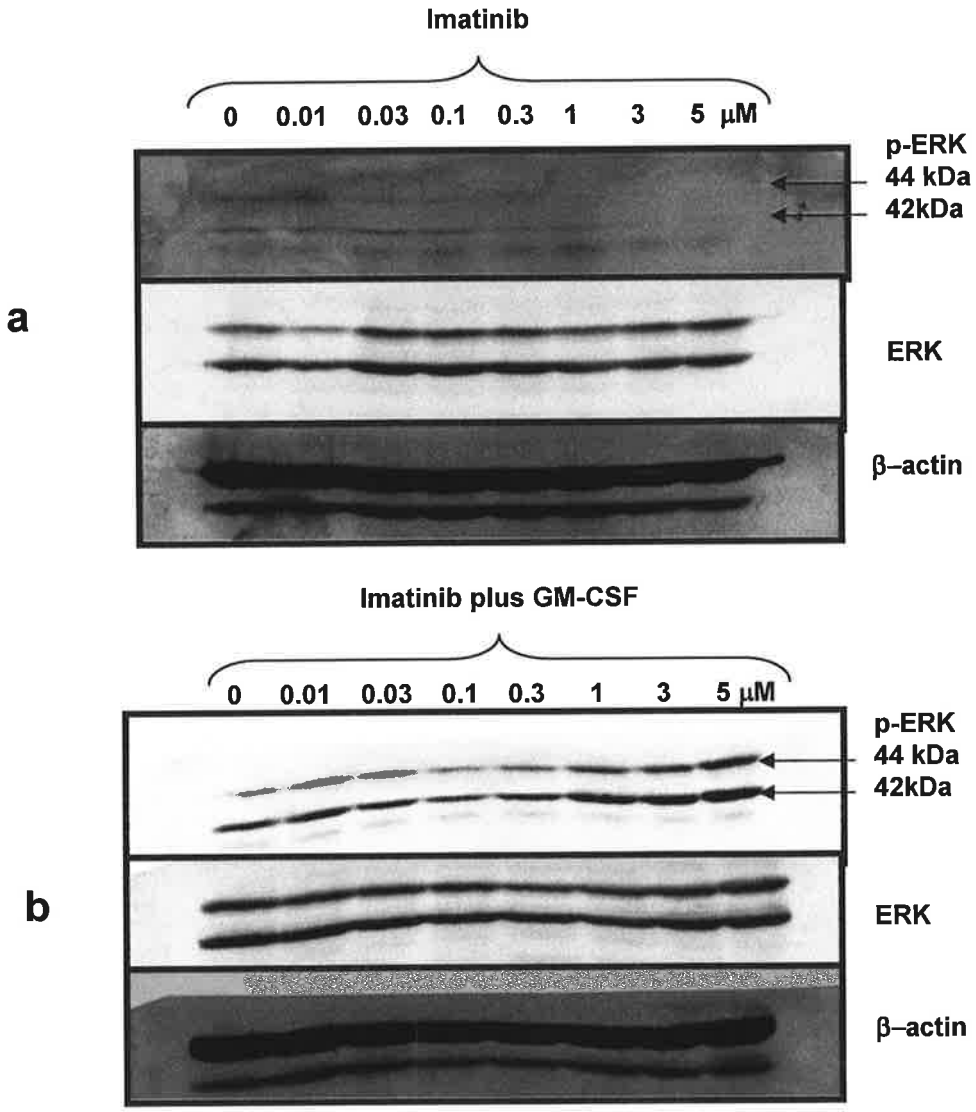

a
Imatinib
0 0.01 0.03 0.1 0.3 1 3 5 μM
p-ERK
44 kDa
42kDa
ERK
β–actin
b
Imatinib plus GM-CSF
0 0.01 0.03 0.1 0.3 1 3 5 μM
p-ERK
44 kDa
42kDa
ERK
β–actin

Figure 6.8

The level of p-Akt was not reduced as seen in Figure 6.9. It became increased at 10 μM which was correlated with the increased binding of p-Akt to Bim and Bad. Phosphorylated Bad (Ser 136) binding to p-Akt was also increased as well when GM-CSF was added to cultures. P-Akt was increased while the binding of 14-3-3 to p-Akt was not changed. This may be associated with the decreased binding between 14-3-3 and p-Akt because the level of p-Akt was increased while the 14-3-3 binding was not changed. The level of p-Akt binding to 14-3-3 was also observed in Figure 6.5 which the density of p-Akt was not reduced along with increasing concentration of imatinib. These two different data confirmed each other that Akt became re-activated at higher concentrations of imatinib. That is firstly, p-Akt level was increased by imatinib and secondly, the binding of 14-3-3 to p-Akt reduced using imatinib as well. These two results suggested p-Akt was re-activated and potentiated to bind with other protein such as Bim or Bad inhibiting their function. This was confirmed by probing the same Akt-immunoprecipitate blot with anti-Bim and Bad antibody, which showed that Bim and Bad level was increased as well. The level of p-Akt increased with added GM-CSF compared with non-treated cells. Again, both Bim and Bad overall increase the binding to Akt when GM-CSF was added, compared with non-treated cells.

6.4.4.6. *Bim was showed binding to other proteins which were associated with apoptosis.*

To examine the protein binding to Bim, cell lysate was immunoprecipitated using anti-Bim antibody and then the bound protein was resolved on 10% SDS-PAGE and transfered to PVDF (Figure 6.10)

As expected, the level of Bim_{EL} was increased associated with the level of imatinib. At 30 minutes evaluation, Bim and its isoforms were increased compared with the non-treated cells. GM-CSF added to imatinib cultures at 30 minutes could increase the isoforms of Bim_{EL}. Even at 10 μM imatinib, although the level of Bim_{EL} was generally decreased, the phosphorylated forms of them remained, compared with the non-treated cells. At 4 hours, the trend of Bim expression in cultures with imatinib only was increased and then overall Bim was decreased again, but still retained its phosphorylated isoform at a higher level than non-treated cells.

Figure 6.9. The increased binding of p-BAD (ser 136) and Bim to Akt when adding GM-CSF to the cultures with imatinib was compatible with the cell survival in the MTS assay (Figures 6.1 and 6.3).

Cell lysate from CTLEN/BCR-ABL was immuno-precipitated with mouse monoclonal anti-Akt Ab having shown that Akt could bind with other proteins which related to the phosphorylation of those proteins. P-BAD (panel a) and Bim(panel b) were increased the binding to Akt which was compatible with the increased p-Akt (Ser 473) as well (Panel c). Interestingly, 14-3-3 could bind with the Akt as well, which was related to the the serine phosphorylation on Akt. and the level of 14-3-3 was not changed among the treatments (panel e). Akt antibody was used to reprobe the same blot of p-Akt to examine the amount of Akt loading on SDS-PAGE gel

14-3-3 was not significantly reduced in this blot although cells received a high concentration of imatinb. 14-3-3 for p-Akt may function as the bridging protein to collect other proteins to associated with it and also function in protecting 14-3-3 from dephosphorylation.

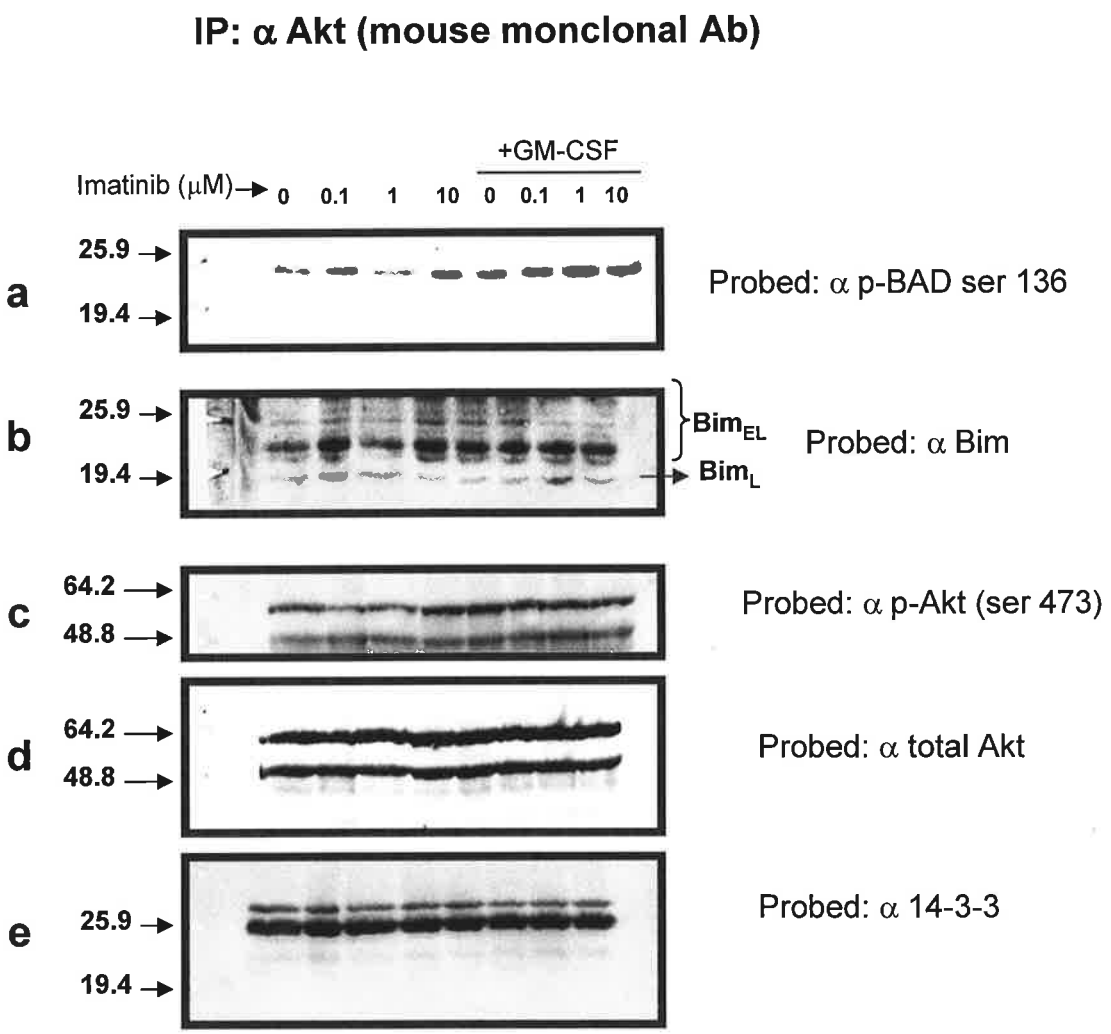

IP: α Akt (mouse monclonal Ab)
+GM-CSF
Imatinib (μM)→ 0 0.1 1 10 0 0.1 1 10
a
25.9
19.4
Probed: α p-BAD ser 136
b
25.9
19.4
Bim$_{EL}$
Bim$_{L}$
Probed: α Bim
c
64.2
48.8
Probed: α p-Akt (ser 473)
d
64.2
48.8
Probed: α total Akt
e
25.9
19.4
Probed: α 14-3-3

Figure 6.9

Bim definitely bound to p-Akt as seen in the experiment where Akt was immunoprecipitated. At short time points, p-Akt increased its binding to Bim with addedGM-CSF and imatinib, the same as was found in Akt immunoprecipitation experiments. When imatinib was used longer for 4 hours, the binding was markedly reduced. The binding of p-Akt and Bim was reversed when GM-CSF was added in cultures with imatinib at 4 hours.

Imatinib also induced the interaction of Bim with Bax. This binding was seen at 4 hour incubation with imatinib but was not very different at 30 minutes of incubation. The Bim/ Bax interaction was reduced when GM-CSF was added.

Imatinib markedly induced Bim / Bcl-XL binding as well. Bim was separated from Bcl-XL by using GM-CSF. This pattern of interaction was seen from only 30 minutes after treatment, however at 4 hours, the binding of Bim to Bcl-XL became increased with GM-CSF.

Erk1/2 (Erk 44/42) is a MAPK family member which involved in the cell proliferation and apoptosis. Erk 1/2 it was activated at very low levels in this cell line, however adding GM-CSF could markedly increase the level of activation and it seemed to increase more in the presence of ≥1 μM imatinib. Erk 1/2 could be detected at a very low level in the wt-BCR-ABL / CTLEN binding with BIM. With GM-CSF, the level of activated Erk1/2 binding with Bim is increased as well. The increased level of Erk1/2 activation on Bim immunoprecipitate blot corresponded to the level of imatinib after 30 minutes of treatment. However, activated Erk 1/2 binding with Bim at 4 hours of culture could not be detected.

6.4.4.7. *GM-CSF also induced the translocation of BAD from mitochondria to cytoplasm.*

The wt-BCR-ABL/CTLEN cells were also cultured in serum starved medium for 6 hours with or without imatinib 1 μM and then incubated at 37 °C in humidified incubator set at 5%CO_2. The cultures were duplicated for the GM-CSF treatment. Ten minutes before harvesting, GM-CSF was added and incubated in the same incubator. Then cell reaction was stopped by cold PBS. The cells were washed twice in cold PBS before starting

Figure 6.10. Immuno-precipitation of the cell lysate derived from the wt-BCR-ABL/ CTLEN with anti-Bim antibody showed that a number of proteins interact with Bim and may alter Bim function.

The wt-BCR-ABL/ CTLEN cells were cultured as described in the text. Anti Bim antibody was used for immunoprecipitation followed by probing with specific antibodies to examine the association of other proteins

The level of Bim_{EL} was increased when imatinib was increased although the difference in densitrometry of Bim_{EL} was not much different at 30 minutes, and became clearer difference at 4 hours. GM-CSF could reduce Bim_{EL} even in the cells treated with imatinib, and the change in Bim_{EL} level was found at both 30 minutes and 4 hours in GM-CSF treatment. However, at 30 minutes, its level became higher and the phospho-isoforms became higher as well when the cells were treated with imatinib and GM-CSF. At 4 hours, GM-CSF reduced overall level of Bim compared with the cells treated with imatinib only. However, its level in the GM-CSF treated cells was not much lower than the non-treated cells suggesting both phosphorylated isoforms and non-phosphorylated isoforms were still preserved although the cells had imatinib in cultures. The level of 14-3-3 was not changed suggesting that the serine phosphorylation of Bim might occur at many sites on Bim and all sites might enhance the binding of Bim to 14-3-3. The different levels of expression of other proteins, such as p-Akt and p-Erk, associated to Bim means the different levels of Bim phosphorylation by those proteins. This suggests that Bim contains many sites of phosphorylation. The preserved level of Bim phosphorylation in the cells treated with imatinib plus GM-CSF suggested imatinib might inhibit Bim degradation by proteosomes.

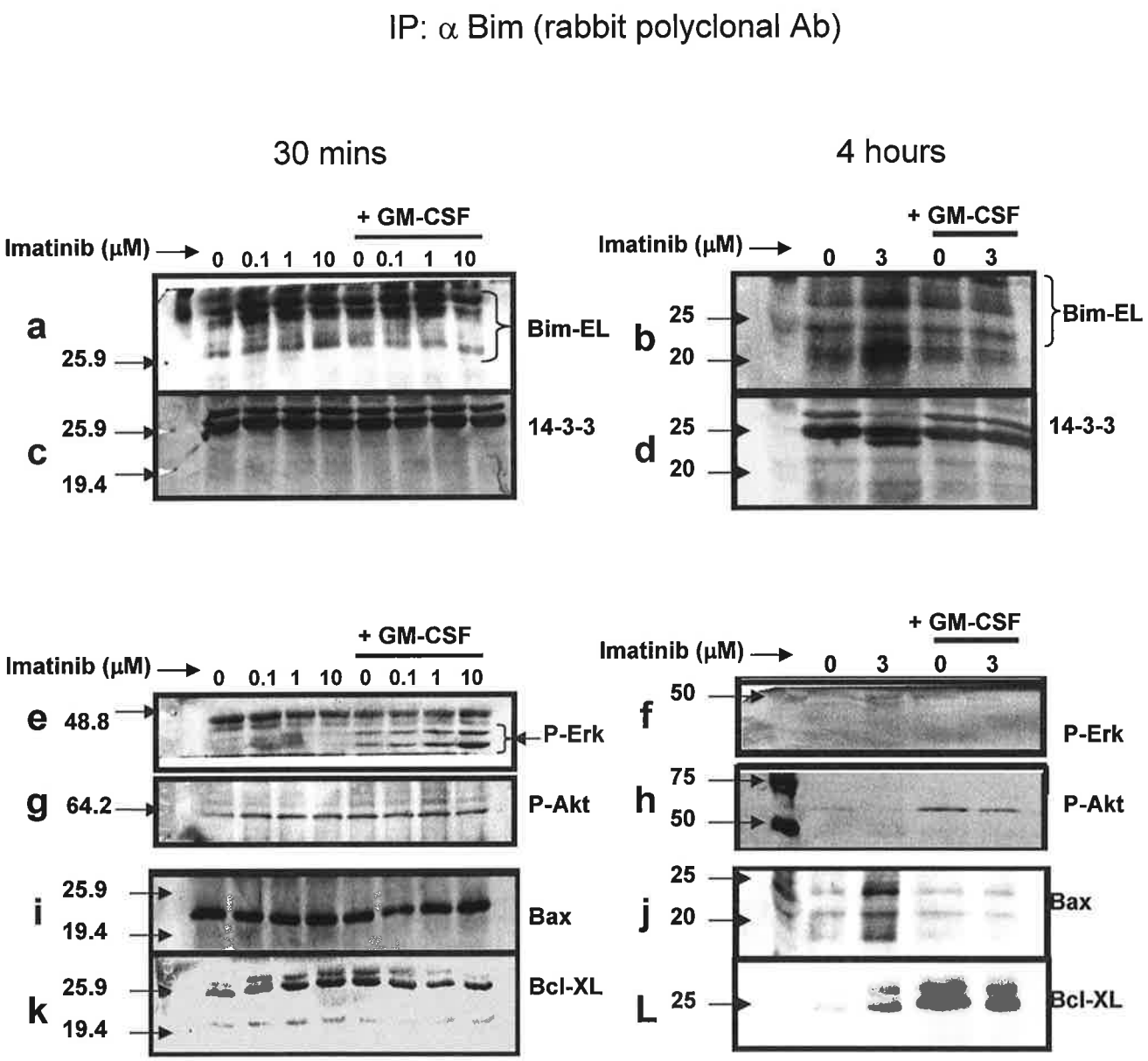

IP: α Bim (rabbit polyclonal Ab)
30 mins
4 hours
+ GM-CSF
Imatinib (μM)
0 0.1 1 10 0 0.1 1 10
0 3 0 3
a
b
c
d
e
f
g
h
i
j
k
L
25.9
19.4
25
20
48.8
50
64.2
75
Bim-EL
14-3-3
P-Erk
P-Akt
Bax
Bcl-XL

Figure 6.10

Figure 6.11. GM-CSF induces the translocation of Bad from mitochondria to cytoplasm in the wt-BCR-ABL/CTLEN.

The cells were cultured for 6 hours in minimised-serum medium. Then a set of cell cultures had 10ng/mL GM-CSF added 10 minutes before harvesting. Then cells were fractionationed as described in the special method. In the Figure, C is cytoplasmic fraction and M is mitochondrial fraction. Imatinib at 1 μM could change the distribution of BAD to mitochondria because the level of BAD (lower band ~ 25 kDa) in M-fraction is found at a higher level than the control, the cells without treatment. GM-CSF could reduce the mitochondrial Bad both with or without imatinib compared with control.

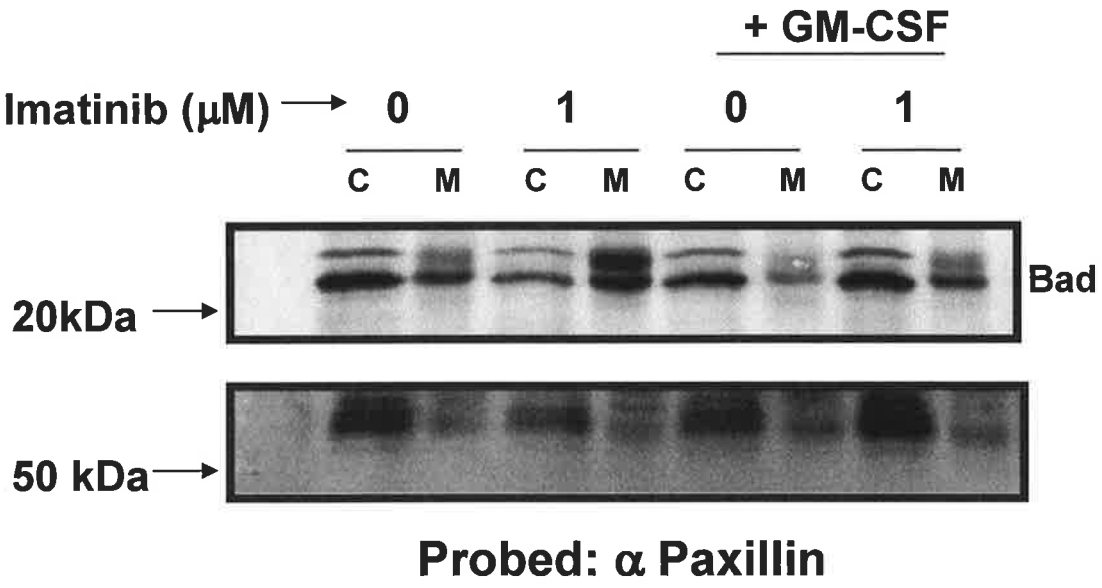

+ GM-CSF
Imatinib (μM) → 0 1 0 1
C M C M C M C M
20kDa →
Bad
50 kDa →
Probed: α Paxillin

Figure 6.11

fractionation as described in the material and method. Equal amount of protein from cytoplasmic and mitochondria fractions were loaded on 10% SDS-PAGE.

Imatinib was shown to reduce the cytoplasmic BAD but increased the mitochodrial BAD compared with the control (cells without treatment). However, this effect was reversed with added GM-CSF suggesting that more phosphotylated Bim was increased. The site of fractionation was proven by anti Paxillin Ab which will bind to paxillin protein in the cytoplasm.

6.5. Discussion

In this study, a number of cell lines were evaluated for their proliferation in response to GM-CSF using the MTS assays in order to choose an appropriate cell line for examining the apoptotic pathways downstream of the GM-CSF receptor and BCR/ABL stimulation. Although KU812 could enhance the effect of BCR/ABL function with added GM-CSF suggesting that this cell line had GM-CSF receptors, the activation through the receptor did not fully replace the function of BCR/ABL when it was blocked by imatinib, in contrast to what is seen in the primary CML cells. This might be due to the strong activity of BCR/ABL which suppresses the expression of the receptors, which is compatible with the finding of Donato *et al.* (Donato, *et al* 2001), or reduce the signalling of the cytokine receptor which was found by Keshan *et al.*(Keeshan, *et al* 2002). On the other hand, the threshold number of the GM-CSF receptors might not be enough for the amount of GM-CSF *in vitro*.

Among those lines examined, wt-BCR/ABL transfected CTLEN containing the human GM-CSF receptor has the full activity of BCR/ABL which could not be overridden or enhanced by GM-CSF. It also contains large number of GM-CSF receptors because the receptor genes are under strong promotor construct. Titration of GM-CSF in the presence of imatinib showed the BCR/ABL function in this cell line could be replaced completely by the activation of GM-CSF receptor. This cell line was used to investigate the mechanisms involving the protection of cells by GM-CSF in the cultures with imatinib.

The ability of GM-CSF to replace the function of BCR/ABL after blockade by imatinib paralleled the responses of primary CML cells in chronic phase to GM-CSF in the cultures with imatinib shown in Chapters 4 and 5. The high levels of BCR/ABL did not change the susceptibility of cells to imatinib as the cells had a similarly low IC50 to that found in primary CML, as demonstrated in the MTS assay of response (Figure 6.3 , panel a-c), although cytokine activation did not result in additional proliferation over control cells.

The findings of primary CML cells in chronic phase were in contrast to this cell line. As primary CML can respond to cytokine stimulation with augmented proliferation, it is apparent that BCR/ABL activity is less extreme than seen in cell lines with BCR/ABL expression under strong promotors. The cells still have partial dependence on cytokines because of cytokines binding primary CML cells may provide additional activation of BCR/ABL substrates and also stimulate other substrates which are not targeted by BCR/ABL. The pattern of response of this cell line to GM-CSF and imatinib is similar to the CML $CD34^+$ cells in response to bystander effect described in Chapter 5. This suggested that there are some proliferation signals that the BCR/ABL of primary CML did not activate and other cytokines or tyrosine kinase receptors are needed to complete full activation and get the same level of proliferation as is seen in the cell lines. This full activation from BCR/ABL activity in the cell line could be fully suppressed with imatinib. As full activation in primary CML cells results from the effect of the combination of cytokine signalling and BCR/ABL, imatinib only inhibits the signals activated through the blockade of BCR/ABL, and activation through cytokine receptors was not affected. Why the BCR/ABL cell lines and primary CML cells in chronic phase are different in this respect is not fully understood, but it may relate to the total amount of BCR/ABL protein per cell.

The data from the MTS assay using fixed imatinib at the concentration of 3 μM showed that imatinib at this concentration could completely block the cell proliferation (Figure 6.3). A quite high concentration of GM-CSF of > 1,000 pg/mL was required to replace the BCR/ABL function to stimulate the same degree proliferation as non-treated cells.

This was a GM-CSF specific effect as it could be fully blocked using the GM-CSF antagonist E21R as seen in Figure 6.1, panel c. This helped to determine the appropriate

concentration of GM-CSF used in the Western Blot experiment for this particular cell line which was able to replace the function of BCR/ABL.

Although E21R could block the effect of GM-CSF as seen in Figure 6.3, panel c when combining GM-CSF and E21R together and adding to the cultures with imatinib, E21R could inhibit cell growth to the level of imatinib only cultures. However, E21R itself could partially promote the survival of this cell line as seen in Figure 6.3, panel a. when imatinib and E21R was combined in the cultures compared with imatinib alone, suggesting it is not completely signalling deficient.

In support of this, we also saw such activation by E21R in primary CML cells in the colony assay in Chapter 4. Figure 6.1 showed that the level of OD value of cells treated with imatinib plus E21R plus GM-CSF in panel c. was not as low as the level of cells treated with imatinib plus GM-CSF in panel a. when the concentration of GM-CSF was 0 - 1000 pg/mL. This suggested E21R may recruit both α and β GM-CSF receptors but the affinity may not high enough to cause the conformation change of GM-CSFβ receptor requires for activating the signals downstream(Bagley, *et al* 1997, Woodcock, *et al* 1999). This is because the β-common receptor alone has no signalling activity and needs association with the α-specific receptors for signalling(Bagley, *et al* 1997, Woodcock, *et al* 1999).

CML cells express a number of anti-apoptotic proteins including proteins in the Bcl-2 family such as Bcl-x_L, Mcl-1 and Bcl-2 (Aichberger, *et al* 2005, Amarante-Mendes, *et al* 1998, Gesbert and Griffin 2000, Horita, *et al* 2000, Nieborowska-Skorska, *et al* 2002, Ravandi, *et al* 2001). However, the contribution of these proteins to inhibition of apoptosis has not been clarified. Bim (Bcl-2-interacting mediator of cell death) is the pro-apoptotic protein in the BH3- only sub-family that also includes Bad and Bid (Gross, *et al* 1999, O'Connor, *et al* 1998, Puthalakath and Strasser 2002). This protein was originally described as a Bcl-2 binding protein that bound to and counteracted the function of Bcl-2 and Bcl-x_L in various cell lines (O'Connor, *et al* 1998). Now it has been identified as the major protein inhibiting other anti-apoptotic proteins in the Bcl-2 family (Puthalakath and Strasser 2002). Primary CML cells have been reported to have the lower expression of Bim

than normal bone marrow cells and re-expression of Bim in CML after treatment with various pharmacological agents correlated with cell survival(Kuribara, *et al* 2004). This suggested that the re-expression of Bim was another attractive strategy to counteract anti-apoptotic mechanisms in CML cells. Bim was measured in this thesis to examine the mechanisms of escape from apoptosis induced by imatinib. Our findings show that Bim may be increased with increasing concentrations of imatinib and addition of GM-CSF decreased levels of Bim but increased the total phosphorylation form of Bim when combined with imatinib, suggesting the degradation of Bim is reduced in cultures with imatinib.

It is clear that imatinib could induce the production of Bim_{EL} in a time dependent manner whereas GM-CSF signalling reduced Bim_{EL} levels over the same time-course. The increased Bim_{EL} level has seen in both 30 min and 4 hours suggesting that imatinib induced both Bim production and degradation. At short time incubation, the many isoforms of phosphorylated Bim_{EL} were increased at greater than 0.1 μM imatinib plus GM-CSF, suggesting that imatinib could inhibit the degradation of Bim_{EL} and enhance the effect of GM-CSF receptor signalling. The cells which were treated with 10 μM imatinib plus GM-CSF showed overall reduced level of Bim_{EL} potentially because of the decrease in production. However, the isoforms of Bim_{EL} found in cells treated with 10 μM imatinib remained higher than in non-treated cells and more phosphorylated isoforms were found compared with cells treated with GM-CSF only, suggesting imatinib could inhibit degradation at high levels. This is supported by the binding of Bim to p-Akt, which was increased at 30 minutes (Figure 6.10, panel a). At 4 hours, the binding of Bim to p-Akt was also increased in the GM-CSF treated cells, suggesting the phosphorylated isoformed were increased with GM-CSF, although reduction in binding activity at 3 μM imatinib could be from the reduced production of Bim. We believe that p-Bim was not much reduced because the binding of Bim to p-Akt was still remained higher than non-treated cells. These data suggested that imatinib may inhibit proteosome degradation via a non-BCR/ABL dependent pathway when used at high level.

The reduced Akt binding to Bim at 4 hours in the cultures without GM-CSF might result from both reduced Bim production and increased Bim degradation. This would result in

lower levels of phosphorylated Bim and further reduce the binding of Bim to p-Akt. Imatinib without GM-CSF could induce Bim production, although the binding of Bim to p-Akt became reduced at 4 hours suggesting there may be other mechanism preventing this binding activity including 1) it may be the mechanism through 14-3-3 to bind and prevent p-Akt binding other protein, 2) inhibition of BCR/ABL will inhibit the activation of Akt and 3) imatinib at 4 hours may strongly induce the serine/ threonine phosphatase to de-phosphorylate p-Akt. The added GM-CSF could block all these mechanisms and reverse p-Akt activity.

Bax is one of the major pro-apoptotic proteins. Increasing imatinib concentration is known to induce CML cell apoptosis. If increasing imatinib induced the interaction of Bim/Bax, it meant that the interaction of Bim/Bax might help to enhance the function of Bax. Therefore, the possible hypothesis was that GM-CSF may break this interaction by phosphorylating Bim and inducing Bim sequestration by 14-3-3.

The Bcl-XL is an anti-apoptotic protein of the Bcl-2 family. Again, the interaction of Bim with Bcl-XL mayt inhibit the function of Bcl-XL and promote cell apoptosis. GM-CSF might help to prevent this interaction in a similar fashion to the Bim/Bax interation. The level of binding of Bim to Bcl-XL was increased in culture with GM-CSF, which might be from 1) the threshold of Bcl-XL was increased by p-STAT5 activation which occurred with GM-CSF treatment and 2) the level of de-phosphorylated Bim was increased. The amount of Bim might not be the reason of increased Bim/Bcl-XL binding because Bim was shown to have more phosphorylation with GM-CSF even in culture with imatinib. Therefore, the Bim/BCl-XL binding should be broken by the p-Bim. The increased binding found at 4 hours when GM-CSF was applied may be from the increased production of BCl-XL.

Erk1/2 (or p44/42 MAPK) is a signal which was not strongly stimulated by BCR/ABL in this cell line although a large amount of total Erk was detected by Western Blot. This suggested the pathway through Erk1/2 was compensated by other substrates which were predominantly activated by BCR/ABL. In another words, the BCR/ABL in this cell line might shunt to directly activate the substrates downstream of p-Erk and result in negative feed-back to inactivate Erk (as shown in Figure 6.8). Therefore, activation of this pathway

required strong stimulation by other factors such as cytokines. This is why a high concentration of GM-CSF was required to promote cell survival to reach the same cell survival as non-treated cells (Figure 6.1). The higher Erk activation at higher concentrations of imatinib when re-activated using GM-CSF (Figure 6.8) correlated with cell survival by MTS, suggesting imatinib might inhibit the negative-control of p-Erk. Evidence for this includes the finding that Bim had binding to p-Erk at higher concentrations of imatinib in cultures with GM-CSF.

The increased binding of Erk1/2 to Bim is dependent on the level of Erk1/2 activation in the cells. Using the cell line showed that BCR/ABL was not not involved in its activation, although the level of non-activated Erk was quite high. This is correlated with the level of Bim binding with Erk, as its binding could not be detected without GM-CSF treatment. Added GM-CSF led to the increased p-Erk and also induced the p-Erk to bind with Bim, as shown in Figure 6.4 and Figure 6.10. Levels of non-phosphorylated Erk could not be detected at 4 hours is the same reason as level of p-Akt markedly reduced at 4 hours as well. Potential reasons include 1) p-Erk and p-Akt were activated at very short time and then their level was decreased very quickly by some serine/threonine phosphatase or protease enzyme, or 2) Bim binds with p-Akt rather than p-Erk , 3) Bim when binding with p-Akt will inhibits p-Erk binding and added GM-CSF may allow p-Erk to have chance to bind with Bim, but only for a short time.

The level of p-Akt at 10 μM imatinib, which is generally toxic, was low as assessed by densitometry. However, the total Akt, which was detected by reprobing with the Akt-Ab on the same blot of p-Akt, was very low at high concentration of imatinib suggesting that the total Akt production was decreased. Therefore, the ratio between p-Akt:Akt was analysed. The ratio was increased at a dose-dependent pattern (Figure 6.4, 6.5). The addition of GM-CSF could increase p-Akt level. When Akt was immunoprecipitated and the p-Akt expression of the cultures were compared using the cells treated with imatinib and PMA as the control, p-Akt signal showed increased in cultures with GM-CSF over cells with PMA and showed similar level of p-Akt in cells with imatinib alone to the cells with PMA. This suggests imatinib did not reduce the p-Akt level even at high concentration of 10 μM, and GM-CSF can enhance p-Akt level in the presence of imatinib.

Phospho-Akt was bound by 14-3-3 as well, which may help p-Akt to escape normal serine/threonine phosphatase deactivation. Once imatinib was used, it presumably helped to increase JNK phosphorylation and then the activated-JNK phosphorylates 14-3-3 and separates 14-3-3 from p-Akt. Therefore, this binding is decreased when increasing concentration of imatinib. Cells may be prevented to proliferate due to imatinib but may be spared from apoptosis because of the activity of p-Akt. The p-Akt could activate Bim but only EL-isoform (Meller, *et al* 2006) which is the important form inducing apoptosis by binding to BCL-XL. Cells possibly survive imatinib without proliferation by the separated-Akt activating Bad and preventing Bad migrating to mitochondria (Figure 6.11)

The experiments in this chapter showed that during imatinib exposure of BCR/ABL positive cells, some survival signals were still activated and other signals such as Erk1/2 become activated in the presence of GM-CSF. Some such as Akt were constitutively activated by BCR/ABL and GM-CSF can provide more activation. These mechanisms could help to prevent cells from entering apoptosis through the Bim/ Bad pathway. 14-3-3 binding to p-Akt may be the mechanism to prevent p-Akt from de-phosphorylation but did not prevent the activation of Bim and Bad by p-Akt.

Bim as described above is a major protein regulating cell apoptosis. The regulation of Bim function involves both the Bim level and its levels of phsphorylation. Bim level is controlled by production and degradation, which is exclusively through proteosome activity. Only Bim degradation will reduce all forms of Bim, both phosphorylated and non phosphorylated. Reduced Bim production does not necessarily involve the de-phosphorylation of Bim, therefore levels of phosphorylated forms may not be decreased. BCR/ABL and cytokine receptors, including GM-CSF receptor, reduce Bim production and increase Bim degradation, which will decrease total Bim level. Again, both GM-CSF receptor and BCR/ABL can activate Bim phosphorylation through PI3K/Akt and MAPK pathways which will activate p-Akt and p-Erk to activate Bim directly. Activated Bim will be sequestered by 14-3-3 and LC8 and stop Bim from inhibiting BCL-XL activity and activation of Bax. The decreased Bim level and increased Bim phosphorylation therefore decreased apoptosis. The diagram summarizing these complex interaction is shown in Figure 6.12 (Gross, *et al* 1999, O'Connor, *et al* 1998, Puthalakath and Strasser 2002).

In the absence of signalling, BCR/ABL functions in this anti-apoptotic pathway. Imatinib will block this activity through direct blockade of BCR/ABL. GM-CSF will replace this function by inducing the substrates which are not up-regulated by BCR/ABL, such as Erk, or enhance the substrates which are free from the inhibition of imatinib such as Akt. Imatinib was also found to inhibit proteosome degradation involved by BCR/ABL (Figure 6.6). This correlates with the increase of Bim level and increased cell apoptosis because the non-phosphorylated form is also increased. Added GM-CSF helps to promote cell survival at high concentration of imatinib through the increase phosphorylation of Bim by the activation of p-Akt and p-Erk (Figure 6.12).

Figure 6.12. A model of cross-talk between GM-CSF and BCR/ABL operating through Bim.

This is one of the channels which GM-CSF could share pathways with BCR/ABL. Bim is one of the major signals relating to apoptosis. The level of Bim is controlled by the production and degradation, but the activity of Bim is controlled by phosphorylation process. BCR/ABL is like GM-CSF in terms of control of Bim function. Both of them could phosphorylate Bim through the PI3-K/Akt pathway. Once Bim is phosphorylated, it will be sequestered by 14-3-3 or LC8 (one of the cytoskeleton proteins which induces Bim to localise in plasma membrane). This will separate Bim from the binding to BCL-XL or BAX and inhibit apoptosis. The level of Bim is controlled by both PI3-K/Akt and proteosome activation. PI3-K/Akt will activate some signal transcription such as FOXO and inhibits its nuclear localisation, which in turn inhibits the DNA transcription for Bim. PI3K/Akt also activates m-TOR and induces nuclear localisation of m-TOR. This also inhibits Bim production as well. Proteosome degradation is able to destroy all isoforms of Bim and is activated by both GM-CSF and BCR/ABL.Therefore, Bim will be decreased generally, not just the non-phosphorylated form.

In our study, imatinib could increase all forms of Bim at imatinib doses which completely suppress BCR/ABL function, even in the presence of GM-CSF. Therefore, imatinib may have a universal function to inhibit proteosome degradation. The red lines in Figure 6.12 show how imatinib blocks the pathways relating to the regulation of Bim level and Bim phosphorylation.

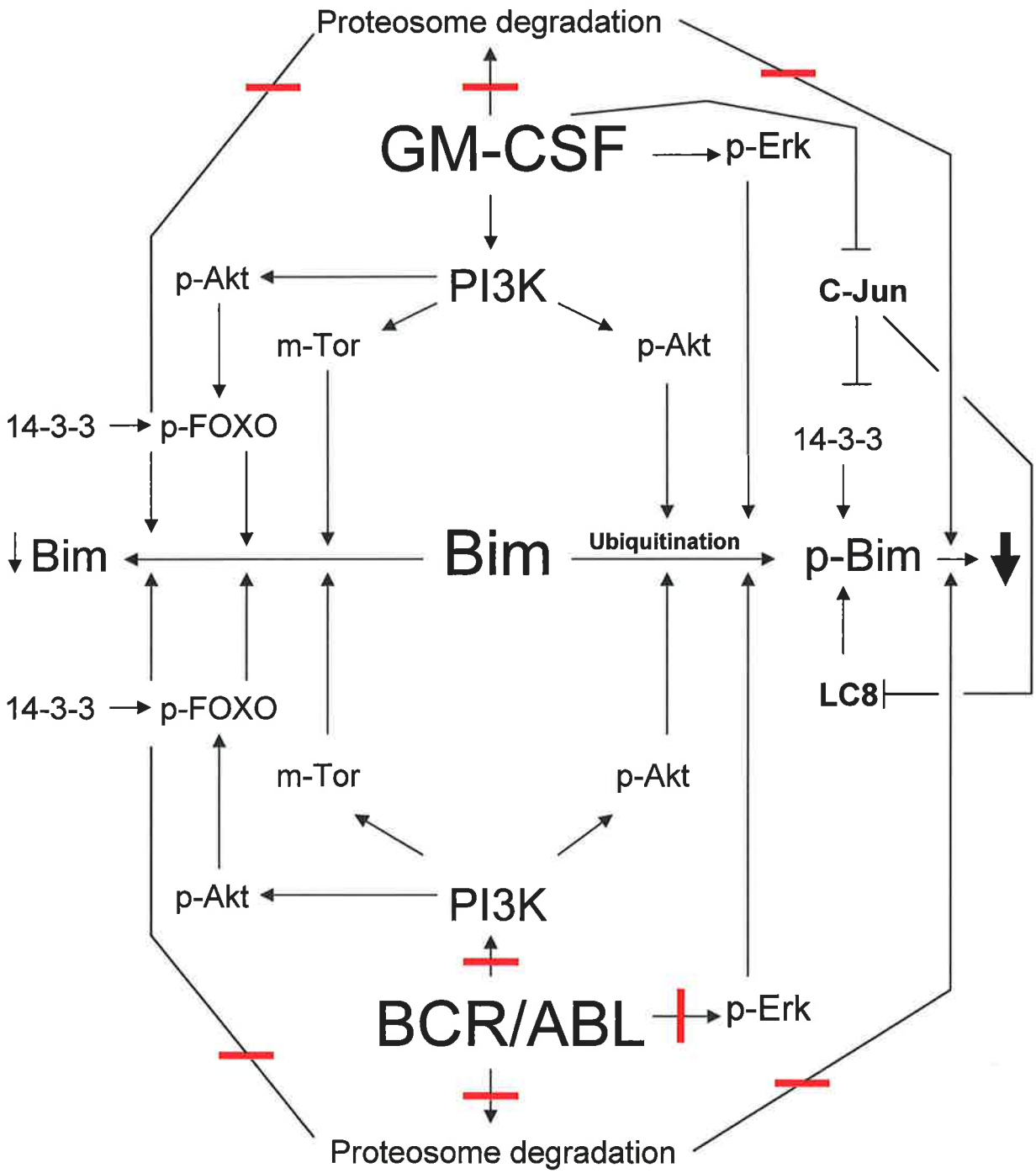

Proteosome degradation
GM-CSF
p-Erk
p-Akt
PI3K
C-Jun
m-Tor
p-Akt
14-3-3
p-FOXO
14-3-3
Bim
Bim
Ubiquitination
p-Bim
LC8
14-3-3
p-FOXO
m-Tor
p-Akt
p-Akt
PI3K
BCR/ABL
p-Erk
Proteosome degradation

Figure 6.12

Chapter 7.

GM-CSF and the apoptosis of primary CML $CD34^+$ cells

7.1. *Introduction*

The wt-BCR-ABL/ CTLEN study in Chapter 6 has shown that these cells, which had high levels of BCR/ABL activity, could not equally activate all the main signaling pathways involving cell survival and apoptosis. This is in contrast to other reports with other transfected cell lines (Daley and Baltimore 1988, Laneuville, *et al* 1991, Pierce, *et al* 1998). Some signaling proteins seemed to be suppressed by the major activation by BCR/ABL such as Crkl. Those signaling proteins such as Akt and Erk which were important for cell survival in general haematopiesis have been shown in Chapter 6 to be marginally activated by BCR/ABL. This cell line contains the complete hGM-CSF receptors (both α-specific and β-common receptors). Therefore, they could freely signal on binding of GM-CSF. This confirmed the previous report that the BCR/ABL-positive cells could spontaneously grow and expand without the addition of cytokines, because even high concentration of GM-CSF at 10 ng/mL could not enhance the spontaneous growth of this cell line(Clarkson, *et al* 2003).

In addition, a high concentration of GM-CSF was required to activate this cell line to reach the spontaneous survival of the cell line in the cultures where BCR/ABL activity was blocked with imatinib. This suggested that to activate the suppressed signals resulting from the major activation by BCR/ABL needed a sufficient threshold level of other factors. For the primary CML cells, the function of BCR/ABL is not as strong and complete as the cell line as seen in Chapters 3 and 4. GM-CSF could enhance or more accurately, provide additive stimulation to that of BCR/ABL which suggested other signals apart from those activated by BCR/ABL are not dominated or suppressed by the activity of BCR/ABL. Therefore, the primary CML did not need a high threshold of GM-CSF to get the same base-line spontaneous proliferation in cultures with imatinib. However, GM-CSF signaling is reportedly based on affinity of receptor, so very high doses of GM-CSF will not result in additional signal(Guthridge, *et al* 1998). Therefore why this cell line needs such a high concentration of GM-CSF is still not understood.

Studies performed several years ago used haematopoietic cell lines or primary murine bone-marrow cells transfected with BCR/ABL to examine BCR/ABL regulation of

proteins involved in proliferation, survival and apoptosis (Clarkson, *et al* 2003). Due to BCR/ABL being under control of very strong promoter in these studies, there was controversy over how well they related to the function of BCR/ABL in primary CML cells. In addition, those studies have also shown that cytokines could not augment the function of BCR/ABL and enhance spontaneous proliferation. The study on the wt-BCR-ABL / CTLEN in Chapter 6 also showed similar results which differed from the primary CML cell findings in Chapter 3 and 4. In the current chapter, signaling in the primary CML $CD34^+$ cells was compared with that of the wt- BCR-ABL / CTLEN cell line after GM-CSF and/or imatinib treatment.

7.2. *Aims of the study:*

7.2.1. To determine if imatinib induces apoptosis in CML $CD34^+$ cells, and if added GM-CSF can prevent apoptosis.

7.2.2. To examine differences in signal transduction between the wt-BCR-ABL / CTLEN cell line and primary CML $CD34^+$ cells in terms of response to imatinib and GM-CSF.

7.3. *Special methods*

7.3.1. *Examination of active caspase expression by flow cytometry using Fluorescent inhibitor of caspases (FLICA) assay*

Flow cytometric analysis of apoptosis was examined by FLICA, fluorecein labeled FAM-VAD-FMK which irreversibly associated to many caspases (capase-1, -3, -4, -5, -6, -7, -8 and -9) marking cells destined for apoptosis. FLICA reagent was supplied as a white lyophilized powder which was dissolved in 50 µL DMSO to each vial to yield a 150x concentrate. This was kept frozen at –20 °C until use. The working solution was prepared by diluting the stock 1:5 (diluted to 30x) in PBS at pH 7.4.

The viable CML $CD34^+$ cells from 3 CML-patients in chronic phase were sorted using PE-conjugated CD34 Ab and 7-AAD to obtain $CD34^+$/ $7\text{-}AAD^-$ cells. The sorted cells were seeded in 96-well plates at cell density of $5x10^5$ cells/mL in SDM supplemented with 1µM

imatinib and/or 300 pg/mL GM-CSF. The effect of bystander cells was performed using the same number of unselected total MNC in each well as the selected-CD34$^+$ only cultures, but evaluated only CD34$^+$ cells at the time-point of harvesting. Cells were cultured in the final volume of conditioned SDM of 300 µL. Ten micro-millitres of FLICA at 30x or 1:5 dilution was added to each well. Cells were cultured over 3 days and harvested at time-point as indicated in Figure 7.1. The percent FLICA positive cells was calculated from the ratio of CD34$^+$/ FLICA$^+$ cells over the total CD34$^+$ cells. The apoptotic index is the delta of slope of curve of percent FLICA$^+$ cells over the time of interest which allows the rate of the accumulation of apoptotic cells over the time of interest to be determined.

The equation to calculated slopes of the data derived from FLICA assay used the linear correlation program in EXCEL. The slope shown in Figure 7.6 was not the true rate of % change of the increased apoptotic cells and needed to change the value on x-axis to be time of observation as shown in formula in topic 7.4.6. Data shown in figure 7.6 aimed to compare the rate of apoptosis.

7.4. ***Results:***

7.4.1. *CML CD34$^+$ cells in mixed MNC cultures were less sensitive to imatinib. However, addition of GM-CSF did not change cell proliferation and survival.*

To examine the time-course experiment on CML CD34$^+$ cells in cultures with bystander cells, sorted CML CD34 cells using method as described in Chapter 5 were seeded in 24-well plates in SDM supplemented with 1µM imatinib, 300 pg/mL GM-CSF and 10 µg/mL E21R, cells were harvested every 24 hours over 5 days. The cells from this patient have been shown their responses to the bystander in Chapter 5. Although these cells were cultured over 5 days, they still showed that the bystander cells still affect CML CD34$^+$ cells the same as the evidence seen in day 3 time-point in panels a and b. Interestingly, E21R could block this bystander effect as well.

The proliferation figure in panel C clearly showed that CML CD34$^+$ cells in the cultures containing SDM with imatinib, GM-CSF and E21R had the same proliferation activity as

Figure 7.1. Examination of GM-CSF and bystander cell effect om CML $CD34^+$ cell proliferation and viability when exposed to imatinib.

Cells from a single well characterised primary CML sample were examined for dose response and time-course of response to imatinib prior to setting up Western Blotting analysis of proteins associated with proliferation and apoptosis

Panel a (PI) and Panel b (viability) show the response of CML $CD34^+$ cells within the total mononuclear population to imatinib after culture for 3 days, confirming that bystander cells produce GM-CSF which can be blocked by E21R. Further addition of GM-CSF has no additional enhancement to either proliferation and viability.(E21R = 10 μg/mL E21R, GM = 300pg/mL GM-CSF)

The time-course Panel c (PI), d (viability), and e (total viable cell count) examine responses over 5 days. Again added GM-CSF is enable to augment proliferation or survival, but addition of E21R also confirms that production of GM-CSF by bystander cells reduced response to imatinib alone. (No Tx = No treatment, Imatinib = 1 μM imatinib, GM-CSF = 300 pg/mL GM-CSF, GM-CSF/Imat = 300 pg/mL GM-CSF plus1 μM imatinib, GIE = GM-CSF plus imatinib plus E21R)

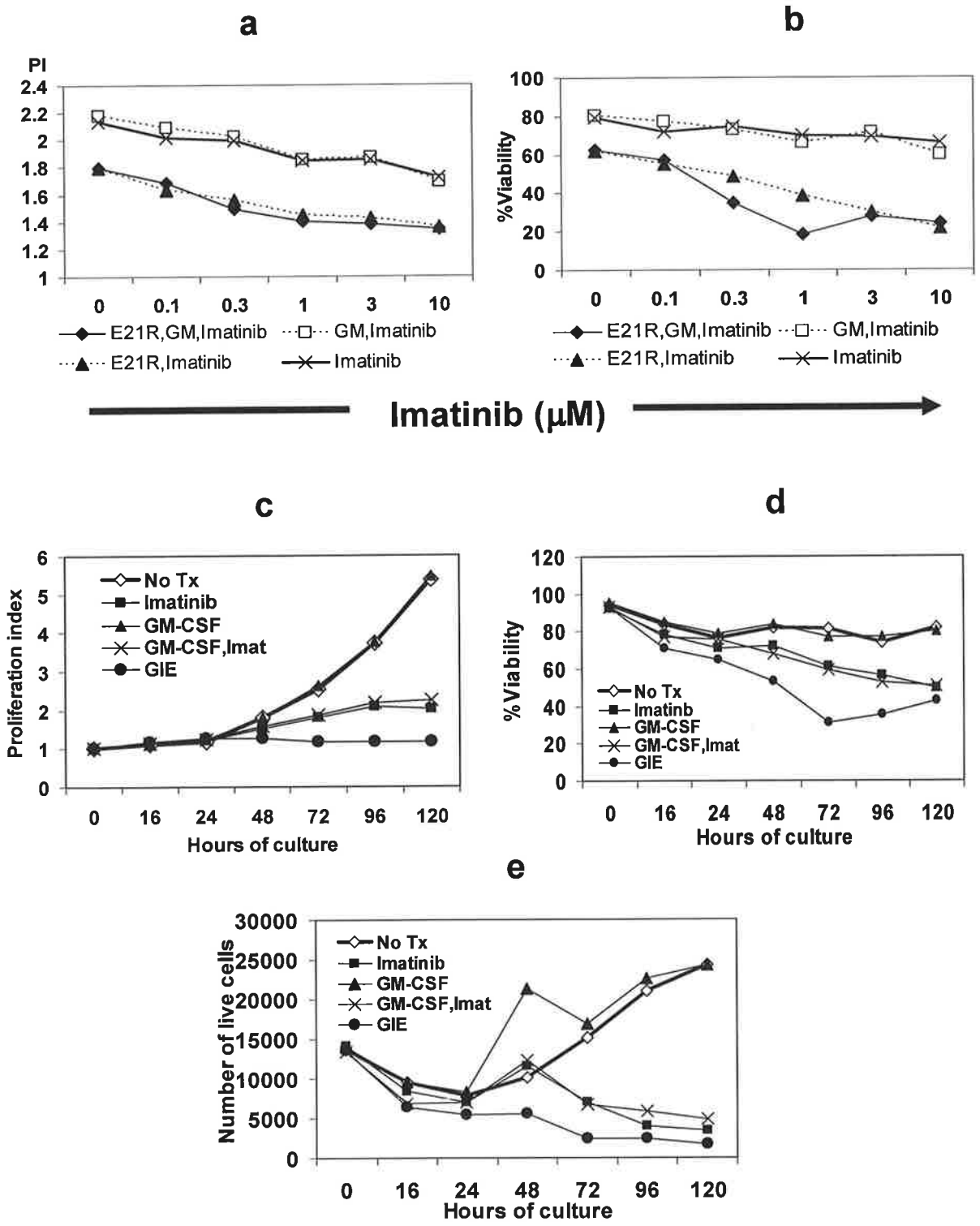

a
b
PI
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
0.1
0.3
1
3
10
E21R,GM,Imatinib
GM,Imatinib
E21R,Imatinib
Imatinib
%Viability
100
80
60
40
20
0
Imatinib (μM)
c
d
Proliferation index
6
5
4
3
2
1
0
No Tx
Imatinib
GM-CSF
GM-CSF,Imat
GIE
0
16
24
48
72
96
120
Hours of culture
%Viability
120
100
80
60
40
20
0
Imatinb
e
Number of live cells
30000
25000
20000
15000
10000
5000
0

Figure 7.1

the cells at time 0, although the absolute number and cell viability by exclusion by 7-AAD showed progressively decrease over the of experiment. Therefore the bystander activity had no effect on this cultural condition in terms of proliferation index. These cells will be tested for their enhanced signaling by GM-CSF later.

7.4.2. *The p-Akt in both primary CML CD34$^+$ cells and CTLEN/BCR-ABL was not reduced by imatinib, but could be enhanced by added GM-CSF.*

Primary CML CD34$^+$ cells from the same patient as in Figure 7.1 were evaluated using Western Blot. Briefly, sorted CML CD34+ cells by using MACS CD34 cells separation beads were starved in SDM for 4 hours to let cells recover from process of preparation. Then duplicated sets $2x10^6$ live cells were cultured in SDM under conditions containing imatinib over the ranges (0, 1 and 3 μM). Then cells were incubated in the 37° C humidified-incubator for 2 hours. Before the end of 2 hours of incubation, one set of imatinib-treated cells had 10 ng/mL GM-CSF added for the final 10 minutes. Then all cells were harvested to obtain cell lysates from $2x10^6$ pelleted cells to which 15 μL of 1% NP-40/TSE lysis buffer was added and incubated on ice for 30 minutes. Then the cell lysates were mixed with 5 μL of 4x loading buffer and boiled for 5 minutes before being resolved in 10% SDS-PAGE gel.

The lysates from cell lines were prepared in a similar way to primary CML cells but only $2x10^5$ cells were used in total volume of 20 μL of cell lysate and cultures were performed overnight using serum starved medium containing 0.5% FCS/RPMI.

The p-Akt which is activated directly by PI-3K had the molecular weight of 60 kDa and was showed the bands as indicated by the makers in Panel a and b in Figure 7.2. The total Akt which was detected to confirm the location of p-Akt has been done using the total Akt Ab to re-probe the same blot as p-Akt. P-Akt level of both primary CML CD34$^+$ cells and the cell line was not reduced during the imatinib treatment and was increased when adding GM-CSF.

To confirm that imatinib could inhibit the function of BCR/ABL, the Crkl Ab was used to probe the same blot as p-Akt (Figure 7.2, panel c). Imatinib could effectively inhibit the

Figure 7.2. Comparison of the p-Akt and p-Crkl expression by primary CML $CD34^+$ cells and the wt-CTLEN/BCR-ABL showed a similar result.

Primary CML $CD34^+$ cells from a patient which has shown strong responses to GM-CSF and bystander effect were sorted using MACS-CD34 isolation beads to get $2x10^6$ cells per 20 μL of cell lysate to load in SDS-PAGE gel. Before cell lysis, cells were cultured in SDM supplemented with imatinib as indicated and/or 10 ng/mL GM-CSF. The density of cell suspension in cultures was about 0.5 – $1x10^6$ cells/ mL. After 2 hour-incubation at 37°C with imatinib only, GM-CSF was added to cultures for 10 minutes and then the cell activity was stopped using 50 mL cold PBS and washed twice. Then $1x10^6$ cells of the primary cells were lysed in 15 μL of 1% NP-40 lysis buffer and incubated on ice for 30 minutes. Then the cell lysate was centrifuged at 16,000xg for 30 minutes at 4°C, and all supernatant was transferred to the new eppendorf before adding 5 μL of 4X loading buffer. The cell lysate from the wt-CTLEN/BCR-ABL was prepared with the same procedure as the primary cells, but using the cell number of $2x10^5$ cells per 20 μL of cell lysate. The cell line were serum starved overnight with 0.5% FCS/RPMI before proceeding to the cell cultures at a density of $5x10^5$ cells/mL in 0.5% FCS/RPMI. Panel a and c derived from primary CML $CD34^+$ cells, and panels b and d from wt-CTLEN/BCR-ABL.

Panel a (primary CML $CD34^+$ cells) showed low level of activated Akt which was not reduced by imatinib and its level was not much increased with GM-CSF. This is compatible with the finding from the cell line in panel b, suggesting activated Akt was remained even in high concentration of imatinib in primary CML cells and this may be the key signal to maintain cell survival without proliferation and potentially to have a proliferation whenever the cells receive optimal stimulation.

The concentration of imatinib used in the cell line is sufficient for inhibition of the activity of BCR/ABL in primary CML $CD34^+$ cells (panel c) and wt-BCR-ABL / CTLEN cells (panel d) which were shown by the inhibition of the expression of phosphorylated Crkl.

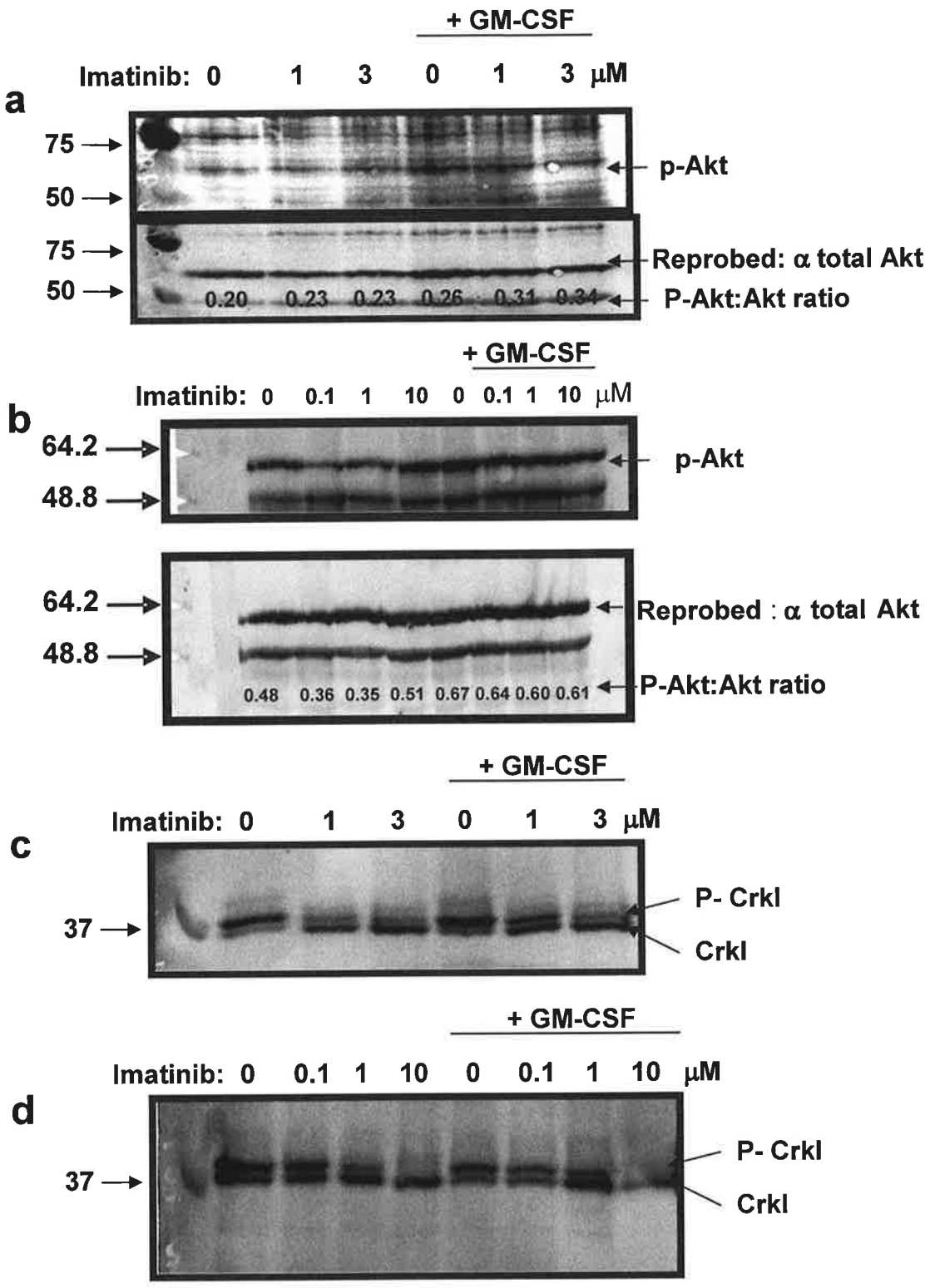

a
+ GM-CSF
Imatinib: 0 1 3 0 1 3 μM
75
50
p-Akt
75
50
Reprobed: α total Akt
0.20 0.23 0.23 0.26 0.31 0.34
P-Akt:Akt ratio
b
+ GM-CSF
Imatinib: 0 0.1 1 10 0 0.1 1 10 μM
64.2
48.8
p-Akt
64.2
48.8
Reprobed : α total Akt
0.48 0.36 0.35 0.51 0.67 0.64 0.60 0.61
P-Akt:Akt ratio
c
+ GM-CSF
Imatinib: 0 1 3 0 1 3 μM
37
P- Crkl
Crkl
d
+ GM-CSF
Imatinib: 0 0.1 1 10 0 0.1 1 10 μM
37
P- Crkl
Crkl

Figure 7.2

phosphorylation of Crkl identified by decreased density of the slow-migrating band with using 1 μM imatinib in both primary CML cells and the cell line. However, when exogenous GM-CSF was applied, the slow-migrating bands became a little reversed at 0.1 and 1 μM imatinib (Figure 7.2, panels c and d). Therefore, the suppression of BCR/ABL by imatinib measured by the reduction of slow-migrating band of Crkl was not much affected by added GM-CSF. The suppression of Ckrl through the reduction of function measured by the decrease of phosphorylated Crkl suggesting imatinib could block the effect of BCR/ABL and this blockade was not able to block the level of p-Akt. This data showed that the imatinib concentration used in this experiment could actually inhibit the activity of BCR/ABL to phosphorylate its target proteins.

7.4.3. *Phospho-Erk expression in CTLEN/BCR/ABL cell line was increased by GM-CSF*

Phospho-Erk, the activated Erk signal in MAPK family was shown to have a low level in CTLEN/BCR-ABL cell line in Chapter 6, but still had some suppresion when the concentration of imatinib was increased. In this Chapter, p-Erk expression in the wt-BCR-ABL / CTLEN was compared to the primary CML $CD34^+$ cells. Without GM-CSF, p-Erk did not show a strong signal but was reduced in density compared with no treatment (Figure 7.3, panel b.). Added GM-CSF did not enhance cell p-Erk expression over non-treated cells, but the level of p-Erk was increased when the concentration of imatinib was increased. In the primary CML $CD34^+$ cells, the level of p-Erk was constitutively strong. Imatinib did not change this constitutive activation and the level was maintained after GM-CSF was added.

7.4.4. *Upregulation of the apoptotic regulatory protein Bim by imatinib*

Bim was shown in the last chapter to be increased with increasing concentration of imatinib which coincided with the decreased survival of the wt-BCR-ABL/CTLEN cells assayed by MTS assay (Chapter 6). In the current experiment we used PMA (Phorbol 12-Myristate 13-Acetate, an analoque of diacylglycerol) which activates protein kinase C (PKC) triggering the phosphorylation of Akt and Erk, as the positive control and cells without treatment as the negative control for Bim production. Then we examined Bim_{EL}

Figure 7.3. Comparison the phospho-Erk expression of primary CML CD34$^+$ cells and wt-CTLEN/BCR-ABL

The cell lysate derived from both types of cells was prepared using the same method as described in Figure 7.2. Panel a was from primary CML CD34$^+$ cells, and panel b and c from the wt-CTLEN/BCR-ABL. The level of p-Erk from the primary CML CD34$^+$ was not much changed when increasing the concentration of imatinib. In contrast to the CTLEN/BCR-ABL, p-Erk seems not much activated but still showed a decreased level when increasing imatinib. Added GM-CSF could change the level of p-Erk according to the level of increasing imatinib. Ras/MAPK pathway may be another pathway for cells to survive imatinib treatment.

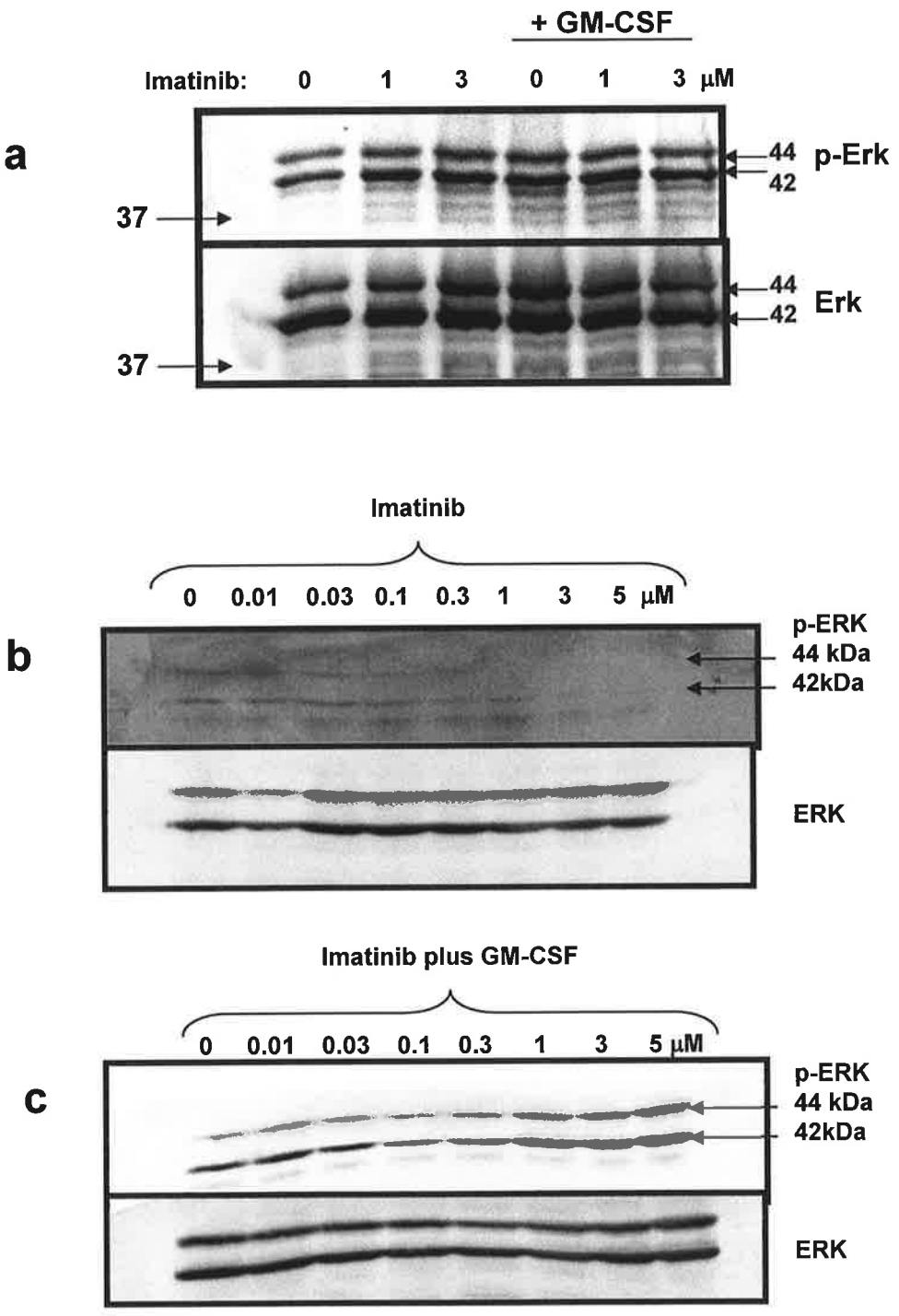

a
+ GM-CSF
Imatinib: 0 1 3 0 1 3 μM
44
42
p-Erk
37
44
42
Erk
37
b
Imatinib
0 0.01 0.03 0.1 0.3 1 3 5 μM
p-ERK
44 kDa
42kDa
ERK
c
Imatinib plus GM-CSF
0 0.01 0.03 0.1 0.3 1 3 5 μM
p-ERK
44 kDa
42kDa
ERK

Figure 7.3

(extra-large Bim) which are believed to be the phosphorylated isoforms of molecular weight ranging from 24 to 30 kDa. Bim_{EL} has been shown to be very important for the regulation of apoptosis. Its non-phosphorylated form can bind with Bax and BCL-XL and inhibit apoptosis.

Figure7.4. panel a shows a Western Blot derived from primary CML $CD34^+$ cells which were treated with imatinib at 0.1 and 10 μM, imatinib plus 50 ng/mL PMA , 20μM Etoposide and 20 μM Etoposide plus PMA. Etoposide was used as a potent apoptosis inducing reagent to examine its effect on Bim. Panel b showed the effect of GM-CSF on the cells treated with imatinib. Again PMA was added to imatinib as the control. The same sample used in panel a. for PMA/imatinib, PMA/Etoposide and Etoposide only was used in panel b. This aimed to ensure that the protein expression was still similar, not coming from the background effect.

Cells were starved overnight in very low serum medium to induce Bim production. Bim was found to have some phosphorylation in non-treated cells. Its phosphorylation was blocked when imatinib was at 1 μM. Then Bim reversed this phosphorylation again when imatinib was 10 μM (Panel a). However, the density of Bim in panel a. seemed generally increased according to the increasing concentration of imatinib.

PMA could enhance the density of Bim and also activate the phosphorylation of Bim. However, when PMA was compared with Etoposide, other isoforms of Bim were found to be increased in density in the cells treated with Etoposide compared to PMA. This was unlikely to be due to experimental variation because when repeated in panel b the expression of Bim was still the same.

Panel b, Bim in cells treated with imatinib/GM-CSF showed similar level to those in imatinib with PMA. However, more isoforms of Bim has been observed in the cells treated with imatinib/ GM-CSF than the cells treated with imatinib only in panel a.

Bim in the cells treated with imatinib/PMA was used to identify the level of Bim of cells treated with imatinib and imatinib/GM-CSF because the same samples were used. The level of Bim treated with imatinib showed higher expression than Bim in imatinib/PMA

Figure 7.4. Effect of imatinib with or without PMA or GM-CSF on Bim expression level and isoforms.

Primary CML $CD34^+$ cells were treated with the condition as described in the figure. Imatinib only could increase the level of Bim but decrease the Bim phosphorylation at 1 mM imatinib suggesting that the phosphorylation of Bim was stopped by the substrates such p-AKT or p-Erk activation. However, the level of phospho-Bim became reversed when the concentration of imatinib reached 10 mM suggesting imatinib at this level could stop the degradation activity rather than the effect of inhibition of p-Akt and p-Erk.

GM-CSF could inhibit Bim production and activate proteosome degradation by observing the overall decrease of Bim level. However, although decreased in total level, GM-CSF did not inhibit the phosphorylation of Bim which could still promote cell survival.

Etoposide could not inhibit phosphorylation activity through BCR/ABL and also it could inhibit degradation activity, similar to imatinib, as seen by the multiple isoforms of Bim.

PMA could activate enhance phosphorylation of Bim and also could not inhibit degradation of Bim as seen in the decreasing isoforms of Bim in PMA treatment compared with etoposide. Etpopside itself could not override the effect of PMA in terms of the degradation of Bim.

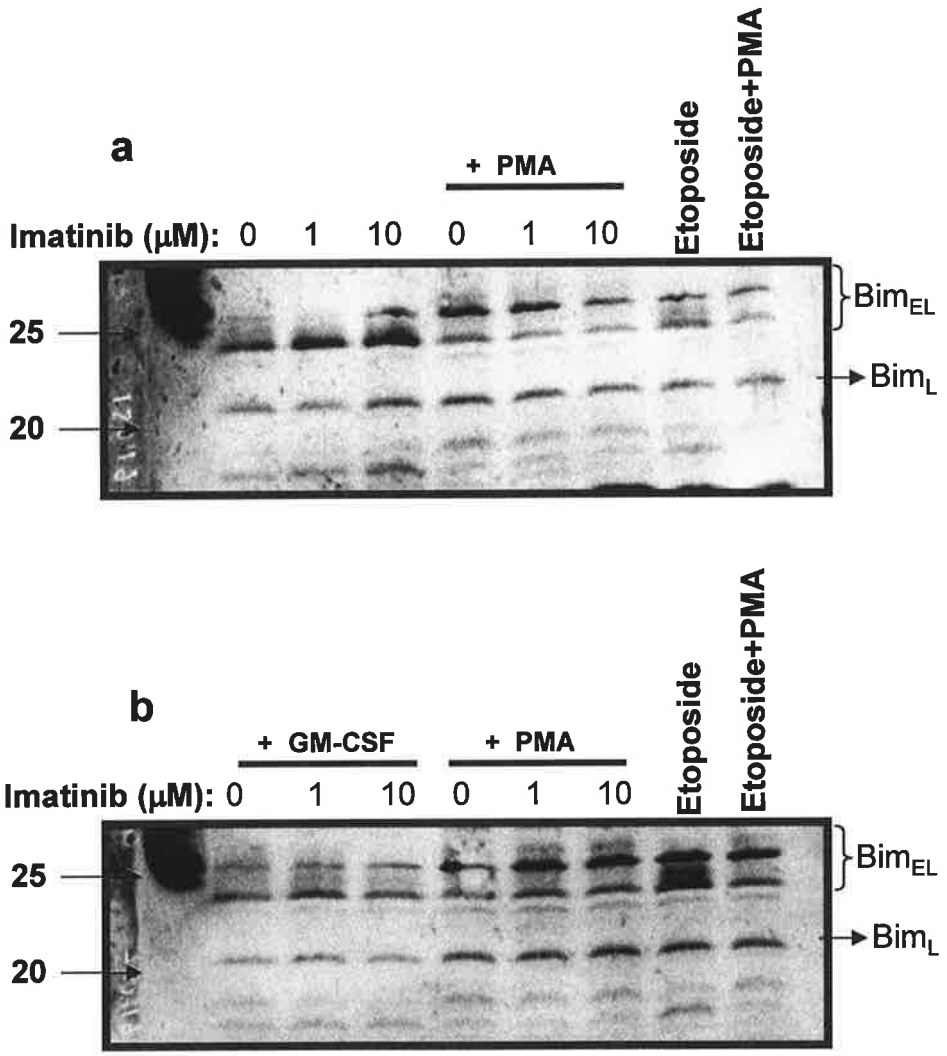

a
+ PMA
Imatinib (μM): 0 1 10 0 1 10
Etoposide
Etoposide+PMA
25
20
Bim$_{EL}$
Bim$_L$
b
+ GM-CSF
+ PMA
Imatinib (μM): 0 1 10 0 1 10
Etoposide
Etoposide+PMA
25
20
Bim$_{EL}$
Bim$_L$

Figure 7.4

cultures (panel a) as described above. However, Bim of cells treated with imatinib/GM-CSF showed similar level to the cells in imatinib/PMA cultures (panel b.). This suggested that Bim was reduced when GM-CSF was added in the presence of imatinib. GM-CSF treatment reduced overall Bim expression but still maintained the multiple phosphorylated isoforms of Bim.

7.4.5. *The total caspase activation in CML CD34$^+$cells induced by imatinib was reduced by the effect of GM-CSF.*

FLICA was added to the cultures at the initiation of cultures in order to detect the accumulation of active-caspase positive cells allowing different conditions to be compared. FLICA itself can inhibit the process of cell-apoptosis because it binds to the intra-cellular active-caspases and then prevents the stimulation of substrates downstream of caspases which result in cell destruction.

CML CD34$^+$ cells were cultured in the SDM with the conditions as indicated in Figure 7.5 and the special method sections in this Chapter. Figure 7.5, FLICA-stained cells could be detected in FL-1 on the flow cytometer. Necrotic cells identified by staining with 7-AAD$^+$ only, were excluded, and the percent of FLICA$^+$ cells within the CML CD34$^+$ cells was determined.

The cultures showed the progressive accumulation of active-caspase positive cells which was induced by 1 μM imatinib, and found the maximal accumulated number was about 70%. Added GM-CSF could abrogate the accumulation of number of caspase-positive cells down to 40% with the percent reduction from the imatinib-only treated cells of about 40%. GM-CSF could prevent induction of apoptosis as well as reduce spontaneous apoptosis during culture.

FLICA data obtained from the mixed-mononuclear cultures showed that the maximal accumulation of the cells in every conditioned culture was ~20% which was lower than the cells destined for apoptosis in CML CD34$^+$ only-cultures treated with GM-CSF alone (~25%).

Figure 7.5. Identification of apoptotic cells using FLICA staining CML $CD34^+$ only cultures and mixed mononuclear cultures.

CML $CD34^+$ cells were seeded in SDM within CML $CD34^+$ only cultures and mixed mononuclear cultures. The total number of $CD34^+$ cells in both groups was equal. FLICA was added to cultures from starting. Cells were harvested to evaluate only CML $CD34^+$ cells at time-points as indicated. The figure shown here was derived from the cells after 24 hours in culture. 7-AAD was used to exclude necrotic cells from evaluation ($7\text{-}AAD^+/FLICA^-$). Their percentage was not much increased during culture under all conditions.

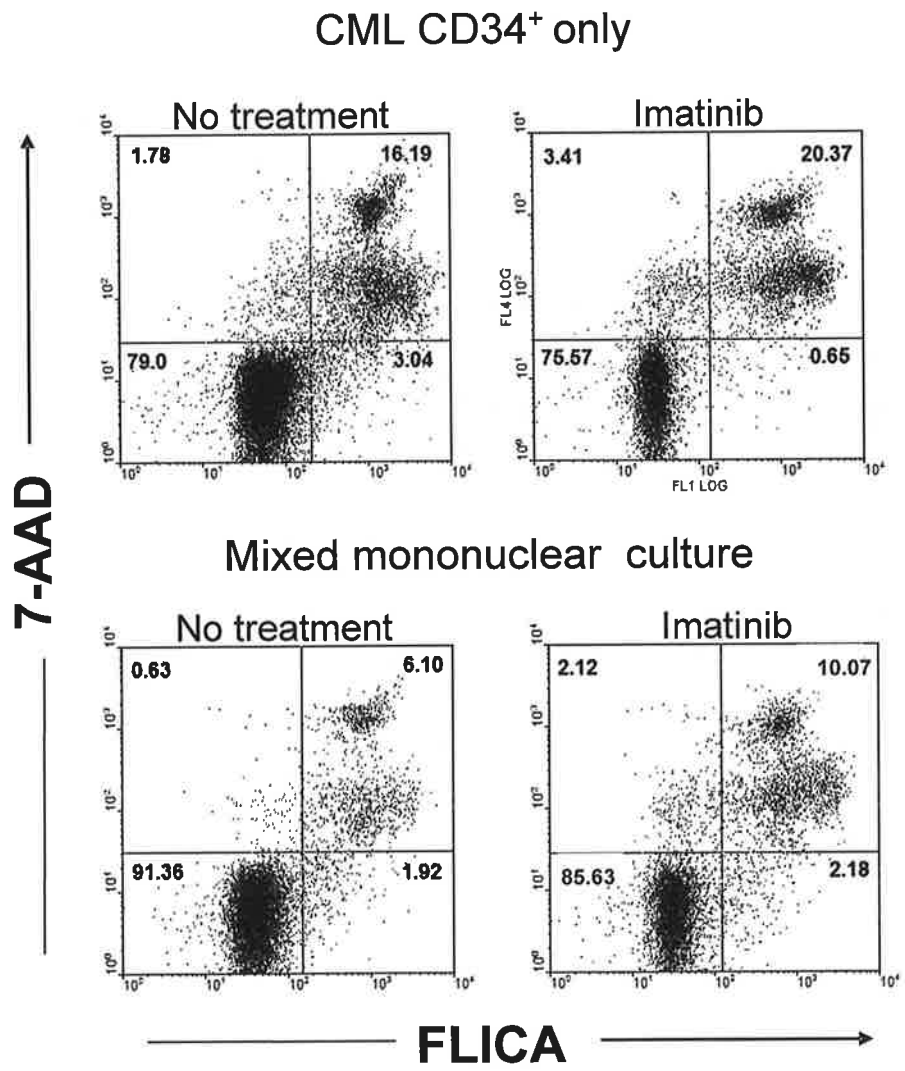

CML CD34+ only
No treatment
Imatinib
1.78
16.19
79.0
3.04
3.41
20.37
75.57
0.65
FL4 LOG
FL1 LOG
Mixed mononuclear culture
No treatment
Imatinib
0.63
6.10
91.36
1.92
2.12
10.07
85.63
2.18
7-AAD
FLICA

Figure 7.5

7.4.6. *The apoptotic index showed GM-CSF had a protective effect on cells treated with imatinib.*

Panel a and b in Figure 7.6 showed the cumulative FLICA-positive cells of CML $CD34^+$ cells from 3 patients in chronic phase which was cultured in the cells containing CML $CD34^+$ cells only. Each point was the mean ratio of FLICA-positive cells to FLICA-negative cells within the total $CD34^+$ cells. One of them was shown to have good responses to the bystander effect and GM-CSF stimulation. Figure 7.1, panel b allowed the average slope of curves in all conditions to be compared. The control was the cells without treatment to show the spontaneous apoptosis. The percent FLICA-positive cells versus hours was counted for the "apoptotic index". The value of slope present in the Figure 7.6 was calculated from the change of percentage within 7 equal unit time-point on axis X. as the formula below:

(Percent of FLICA positive cells at 72 hours – at 4 hours) / 7 time-units = SLOPE

Therefore; (Percent of FLICA positive cells at 72 hours – at 4 hours)

= SLOPE X (7 time-units)

Therefore; Apoptotic index (AI) = SLOPE X (7 time-units) / (72 - 4) hours

Therefore, in panel b, Figure 7.6, the non-treated control cells had AI = 7.4558 X 7 / 68 = 0.767 % /hour (equation 1), AI of GM-CSF only = 3.9575 X 7 / 68 = 0.407 % / hour (equation 2), AI of imatinib treated only was equal to 11.698 X 7/68 =1.204 % / hour (equation 3), and the AI of imatinib plus GM-CSF treated cells = 7.5677 X 7 / 68 = 0.779% / hour (equation 4). The apoptotic index (AI) showed the cumulative rate of apoptotic cells in GM-CSF treated cells in CML $CD34^+$ cells only cultures was the lowest, and this was in contrast to the non-treated cells. GM-CSF could suppress the rate of apoptotic cell production by imatinib down to the same rate as control. However, the pattern of response to GM-CSF with imatinib and the spontaneous apoptotic figure were different. The spontaneous apoptotic figure showed the continuous progression of apoptotic event, but the imatinib plus GM-CSF treated cells showed the slow rate of apoptosis after 48 hours and then reached plateau which meant 0%/hour or no more apoptosis occurred.

Figure 7.6. Examination of GM-CSF and bystander cell effects on apoptosis of CML CD34$^+$ cells using FLICA analysis

Accumulation of apoptotic events over 72 hours culture was measured using the FLICA method (Figure 7.5). Culture of CML CD34$^+$ cells alone and CML CD34$^+$ cells within the total MNC fraction were compared. CML CD34$^+$ cells cultured alone had a higher level of spontaneous apoptosis, which was decreased with the addition of 300 pg/mL GM-CSF. The addition of imatinib markedly increased levels of apoptosis, which could be reduced by added GM-CSF (panel a).

Within the total MNC (panel c), there was markedly less spontaneous apoptosis, and only minor pro-apoptotic effects with addition of imatinib. Addition of GM-CSF was able to halve the spontaneous levels of apoptosis.

Panel b and d showed the equations of the trend straight lines which have the most correlation with the FLICA curves. The slopes of the straight lines was used to calculate the apoptotic index. (equation 1 = no treatment, 2 = GM-CSF, 3 = Imatinib, and equation 4 = Imatinib plus GM-CSF)

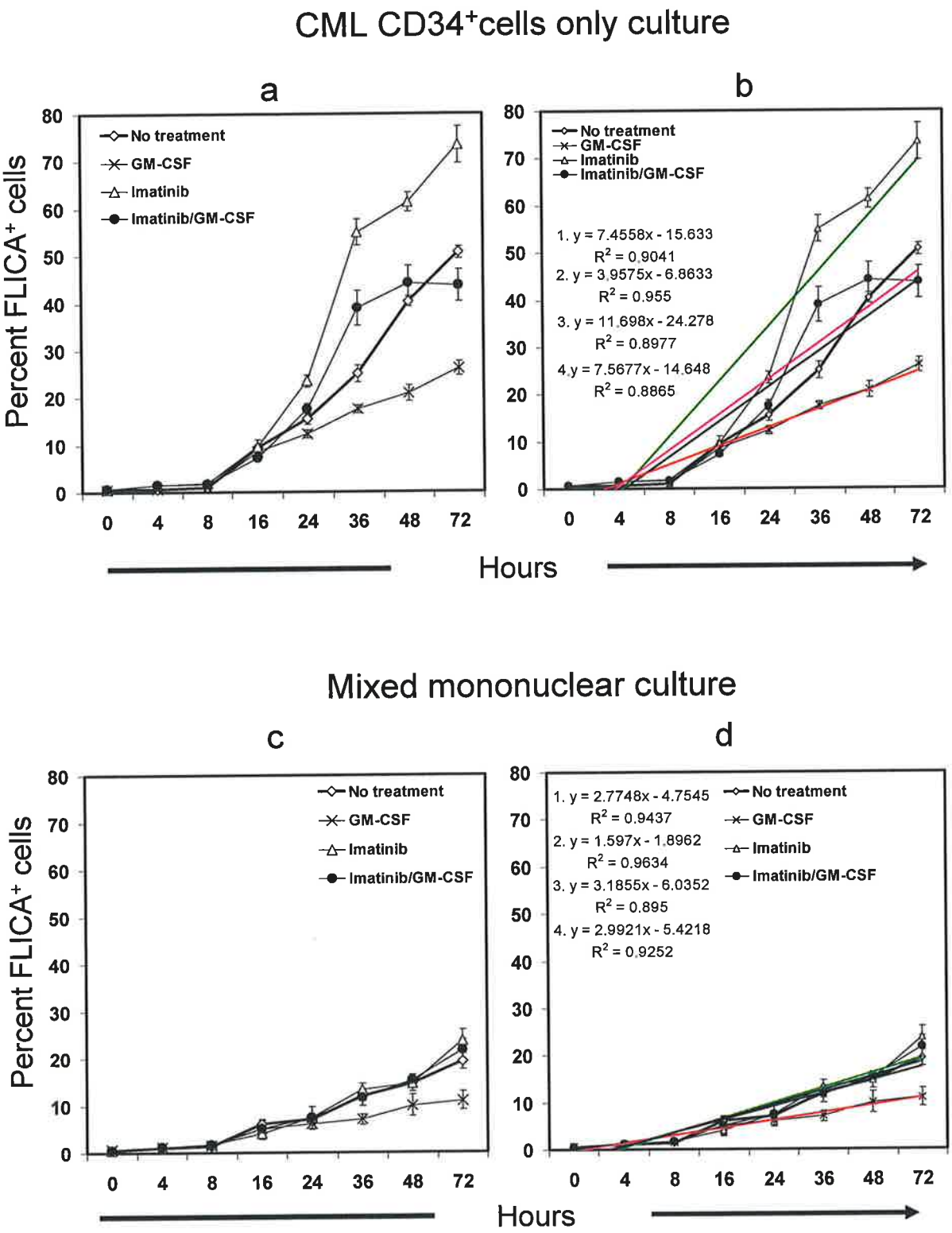

CML CD34+cells only culture
a
b
No treatment
GM-CSF
Imatinib
Imatinib/GM-CSF
Percent FLICA+ cells
1. y = 7.4558x - 15.633
R2 = 0.9041
2. y = 3.9575x - 6.8633
R2 = 0.955
3. y = 11.698x - 24.278
R2 = 0.8977
4. y = 7.5677x - 14.648
R2 = 0.8865
Hours
Mixed mononuclear culture
c
d
1. y = 2.7748x - 4.7545
R2 = 0.9437
2. y = 1.597x - 1.8962
R2 = 0.9634
3. y = 3.1855x - 6.0352
R2 = 0.895
4. y = 2.9921x - 5.4218
R2 = 0.9252

Figure 7.6

In panel c and d in Figure 7.6 showed the cumulative of FLICA-positive cells of CML $CD34^+$ cells which were cultured in the mixed-mononuclear cells under conditions as indicated. The AI could be calculated the same as cells in $CD34^+$ only cultures. In panel d, AI of non-treated cells equated 0.2856% / hour (calculated by using equation 1), AI of GM-CSF only equated 0.1644% / hour (equation 2), AI of imatinib only equated 0.328% / hour (equation 3), and AI of imatinib plus GM-CSF equated 0.3080% / hour (equation 4) (Figure 7.6, equation shown in panel d). All conditioned cultures had nearly the same AI for CML $CD34^+$ cells in mixed mononuclear cultures, except the cells treated with GM-CSF, the lowest AI.

7.5. ***Discussion***:

The bystander effect on CML $CD34^+$ cells was examined to confirm cells which have been shown before to have a good response to GM-CSF could maintain cell proliferation due to GM-CSF produced by non-$CD34^+$ CML cells. Although we had no condition with cells treated with GM-CSF or E21R with imatinib, due to the limitation of the number of purified $CFSE^+$ / $CD34^+$ cells, other arms of the experiment confirmed the role of GM-CSF in the mixed mononuclear cultures. These findings still suggest that the predominant cytokine produced by the bystander cells was GM-CSF and its threshold was as high as the exogenously provided GM-CSF.

At day 5, other cytokines apart from GM-CSF may potentially produced in cultures in sufficient amounts to promote cell survival and proliferation, however, as seen in cells with GM-CSF and no-treatment in Figure 7.1, panel c-e, there is little evidence of cytokines other than GM-CSF. No enhancement of cell proliferation, viability and absolute number of live cells was seen in both cells without treatment and treated with GM-CSF only. This raised questions about the actual cytokines produced in the bone marrow of CML patients. At least three reports showed that GM-CSF was the predominant cytokine found in the serum of CML patients (el-Ahmady, *et al* 1997, Jonuleit, *et al* 1998, Lajmanovich, *et al* 1993) and the other showed this cytokine was predominant in the circulation of mouse model transplanted with CML cells(Zhang and Ren 1998). The unaltered proliferation compared with time 0 in cultures with SDM supplemented with imatinib plus GM-CSF and E21R (in Figure 7.1, panel c.) also strongly suggested that within 5 days of culture,

GM-CSF was still the predomonant cytokine acting on CML $CD34^+$ cells which led to the investigation of the effect of GM-CSF in the apoptosis of primary CML cells.

Although the level of phosphorylation of Akt was not strong, it was constitutively phosphorylated in CML $CD34^+$ cells from this patient. The level of p-Akt was not reduced in cells cultures with imatinib 0-3 μM, suggesting that this protein had other factors to protect it from dephosphorylation after imatinib treatment. This factor may be 14-3-3 owing to the finding that the binding of 14-3-3 to p-Akt (ser 473) was not reduced during imatinib treatment in Chapter 6. From the findings in Chapter 6, the protein 14-3-3 was thought to have quite stable binding to p-Akt and prevent p-Akt from dephosphorylation. The 14-3-3 bound p-Akt might not inhibit p-Akt to activating Bad or Bim, because we found in Chapter 6 that 14-3-3 still binds with p-Akt in the presence of GM-CSF and we know from the previous reports (Keeshan, *et al* 2002) that GM-CSF will phosphorylate Bad through the activation of p-Akt. We assume that 14-3-3 bound p-Akt may help to recruit other signals to associate with p-Akt as seen in Chapter 6 when we immunoprecipitated Bim and still found a stable level of 14-3-3 binding and a stable level of p-Akt(Masters, *et al* 2001). This bridging function of 14-3-3 has been reported previously (Luo, *et al* 1996, Marshall 1996, Xiao, *et al* 1995).

This binding will be prevented by the phosphorylation of 14-3-3 which occurrs when 14-3-3 is activated by c-Jun NH_2-terminal kinase protein(Yoshida, *et al* 2005). The c-Jun N-terminal kinase protein is also activated by DNA-damage (Lei and Davis 2003). The disruption of binding may reduce p-Akt activity because p-Akt will be de-phosphorylated. However, the finding seen in the wt-BCR-ABL/CTLEN cell line in Chapter 6 that the level of binding of 14-3-3 to p-Akt was not reduced in the presence of imatinib, suggesting that imatinib may reactivate the Akt phosphorylation by an unknown mechanism that needs to be elucidated. This helps the GM-CSF receptor to enhance the phosphorylation of Akt and allow cells to escape imatinib induced apoptosis without involving the other pathways stimulated by BCR/ABL

P-Erk, a protein in the MAPK family, can activate both apoptotic and proliferation pathways. Interestingly, p-Erk in this primary CML $CD34^+$ cell sample was not changed or seemed slightly increased when compared with the total Erk after treatment with imatinib.

The increased p-Erk after imatinib treatment was only seen in the cell line in Chapter 6, Figure 6.8 and Chapter 7, Figure 7.5, but only in the presence of GM-CSF. Without GM-CSF, the level of p-Erk is reduced with imatinib. This suggested that p-44/42 Erk (or p-Erk1/2) was constitutively phosphorylated in the wt-BCR-ABL/CTLEN cells. The increased p-Erk with imatinib and GM-CSF suggests that other signals not related to BCR/ABL or not blocked by imatinib activate Erk. In primary CML cells, as the level of function of BCR/ABL is not very high (as seen in MTT assay on the cell lines in this chapter compared with CFSE assay on CML $CD34^+$ cells in Chapter 4), p-Erk might be initiated by BCR/ABL, and then further activated by other alternative pathways. This is an explanation as to why the level of activated Erk was not reduced or not changed after imatinib or GM-CSF treatment. In addition, the nature of human cells (primary CML cells) and mouse cells (CTLEN) was different and might cause different p-ERK activation through BCR/ABL protein as shown in Figure 7.5.

Bim is currently regarded as a major regulator of apoptosis. We observed in Chapter 6 that non-phosphorylated Bim could bind to other substrates in the BH_3-group in the Bcl-2 family (Kuribara, *et al* 2004, Kuroda 2005).. Bim binding to BAX was able to enhance the pro-apoptotic function of Bax, and Bim binding to Bcl-XL was able to inhibit the anti-apoptotic function of Bcl-XL (Kuribara, *et al* 2004, Kuroda 2005).. The phosphorylated Bim will be sequestered by 14-3-3 or LC8 and then Bim will be separated from other BH_3-proteins (Kuribara, *et al* 2004, Kuroda 2005). Therefore phosphorylation of Bim will inhibit its function. We have shown in Chapters 6 and 7 that cells were induced to produce Bim by the deprivation of cytokines or imatinib treatment. In addition, Bim was phosphorylated after stimulation with cytokine. The inhibition of production and the phosphorylation of Bim by cytokines were associated with decreasing function of Bim. Another mechanism which is able to regulate Bim function is proteosome degradation (Akiyama, *et al* 2003, Ley, *et al* 2003). This mechanism can induce Bim degradation and this reduce Bim level in both phosphorylated or non-phosphorylated form. Our finding in primary CML $CD34^+$ cells was compatible with other reports (Kuribara, *et al* 2004). Bim was increased when adding imatinib, but interestingly with imatinib at higher concentrations the level of phosphorylated form increased, suggesting imatinib at higher concentration was able to inhibit the process of degradation of Bim. Imatinib itself would

not induce phosphorylation of Bim. Adding GM-CSF could induce phosphorylation of Bim as many isoforms of Bim increased between molecular 24 – 30 kDa location. However, GM-CSF itself inhibited Bim production and appeared to induce Bim degradation. That is why we found overall decreasing of Bim in the cultures with added GM-CSF. This will enhance effect of GM-CSF in the support of cell survival.

Both PMA and Etoposide increased Bim level and also the level of Bim phosphorylation. The increased Bim by Etoposide may be associated with apoptosis, as found in another study of Bim level and apoptosis in neuronal cells (Biswas and Greene 2002, Putcha, *et al* 2001, Whitfield, *et al* 2001). Etoposide could induce a higher level of phosphorylated Bim compared with the cells treated with imatinib only, imatinib plus PMA and Etoposide plus PMA. This suggested PMA could either inhibit Bim degradation or induce Bim phosphorylation through some signals which have not been identified, suggesting a mechanism for malignant cell survival during Etoposide and imatinib therapy, even though cell division was halted. PMA could not maintain Bim phosphorylation, as we saw the reduction of all isoforms of Bim when adding PMA to cultures. This suggested PMA could induce Bim production and phosphorylation but could not inhibit Bim degradation when comparing cells treated with Etoposide only and Etoposide plus PMA to be compared. A question remains as to the relative importance of decreased degradation, increased Bim production and increased phosphorylation, in the regulation of cell survival. The data showed that imatinib at high concentration could reduce Bim degradation and also increased Bim production (Figure 7.4, panels a and b). Thus Bim phosphorylation was retained due to the reduction of degradation (Aichberger, *et al* 2005). However, the cells still die at high concentrations of imatinib. This suggested that increased Bim level was more important than other factors in the induction of cell apoptosis. However, the potential inhibition of proteosome degradation effect by imatinib at high concentration may enhance the effect of GM-CSF by maintaining overall Bim level and inducing more Bim phosphorylation as we saw the more isoforms of Bim in the imatinib plus GM-CSF treated cells (Figure 7.4, panel b) compared with the cells treated with imatinib only (Figure 7.4, panel a.). This was the further finding to the previous reports (Aichberger, *et al* 2005).

The activation of caspases (Cysteine-Aspartic acid Specific Proteases) is a marker of apoptosis and cell commitment to disassemble the machinery to support cell life(Smolewski, *et al* 2001). It occurrs in response to different inducers of apoptosis, particularly in the mitochondrial induced apoptotic pathways involving the proteins Bad, Bim, Bax, Bcl-XL and Bcl-2 (Smolewski, *et al* 2001). Caspase activation is mostly from the post-mitochondrial inducer which was able to be used as the marker to measure of the activation of the pre-mitochondrial signals. As FLICA marks cells destined for apoptosis and prevent their degradation, it is an accurate marker of accumulated apoptotic events, with less cells lost compared with other methods(Smolewski, *et al* 2001). Apoptosis measured by FLICA in CML $D34^+$ cells in $CD34^+$only culture was compatible with the result found in the CFSE/7-AAD study. (Chapter 5, figure 5.9). Cells with imatinib only had the highest rate of apoptosis with biphasic acceleration, thus a biphasic of cell response to imatinib. The first plateau in Figure 7.6, panel a the highest curve, might result from some cells not entering the cell cycle, with imatinib not affecting these quiescent cells until they entered cycle. GM-CSF could slow the accelerating phase of apoptosis by imatinib, and the character of curve was similar to the spontaneous apoptotic curve except the plateau stage after 48 hours. This might be because GM-CSF at this concentration could help cell survive imatinib by stimulating other signals to maintain viabily However, GM-CSF at this concentration could enhance the effect of BCR/ABL to reduce the spontaneous apoptosis of cells as seen in the cells treated with GM-CSF only, suggesting that BCR/ABL activity alone is insufficient for cell survival in SDM, reflecting previous reports on the partial cytokine dependence of CML $CD34^+$ cells (Goldman, *et al* 1974, Lansdorp, *et al* 1985, Metcalf, *et al* 1974).

The FLICA assay on $CD34^+$ CML cells in mixed mononuclear cultures (Figure 7.6, panel c and d) interestingly showed that the bystander cells protect CML $CD34^+$ cells from spontaneous apoptosis. The added GM-CSF was additive effect for this bystander effect, suggesting other cytokines may be involved in this situation. Although we could not examine the bystander effect by using Western Blot due to cell number limitations, we could conclude indirectly that those signals involved in induction of apoptosis could be inhibited or activated by cytokines produced by the bystander cells, which was predominantly GM-CSF.

Chapter 8.

General discussion

General Discussion

The topic of the research in this thesis was the examination of the response of CML progenitors to GM-CSF, and to determine how GM-CSF modifies the response of BCR/ABL expressing cells to the small molecule ABL kinase inhibitor imatinib mesylate. In addition, non-$CD34^+$ CML cells were identified as a source of GM-CSF able to protect $CD34^+$ CML cells from the action of imatinib.

8.1. *Overview relating to this study*

The $p210^{BCR\text{-}ABL}$ protein is encoded by the BCR/ABL fusion gene in the CML cells. This protein has been studied for several years to determine the role of this protein in expansion of the disease (Donato, *et al* 2001, Laneuville 1995, Mahon, *et al* 2000). It is still unclear whether or not the primary CML cells also require cytokine in the progression of the disease. The primary CML progenitors may require cytokines to aid cell survival in the stressed microenvironment of the marrow. The biology of very primitive CML cells is difficult to study because of their rarity and the lack of specific identifying markers. Some studies has shown that the very primitive BCR/ABL positive cells behave very similar to the normal primitive cells, however, they become mature more quickly than the normal primitive cells. Evidence for this includes the findings that all BCR/ABL positive primitive cells express $HLA\text{-}DR^+$ which indicates committed cells (Holyoake, *et al* 2001). CML progenitors also have an adhesion defect induced by BCR/ABL activity which contributes to premature release from marrow compared with the normal primitive cells. .

The evidence for cytokine signalling involvement in supporting viability of CML cells comes from the study of Willson-Rawls et al. (Wilson-Rawls, *et al* 1996) who reported that BCR/ABL interacts with the IL-3 receptor β-common subunit and constitutively induced its tyrosine phosphorylation. However, the concept of cytokine involvement in the aetiology of CML cell growth is still controversial because although primary human CML cells are not completely cytokine independent, many experiments examining the role of cytokines in CML were performed using transfected cell lines or transfected primary cells in murine models, which may not be applicable to the human disease (Drexler, *et al* 1999,

Hariharan, *et al* 1989, Ilaria and Van Etten 1995, Keeshan, *et al* 2002, Matulonis, *et al* 1995).

8.2. *CML CD34$^+$ cells have spontaneous proliferation due to BCR/ABL which is enhanced by GM-CSF*

In our experiments, we show that primary CML CD34$^+$ cells have spontaneous proliferation whereas normal CD34$^+$ cells do not, as seen in Chapter 3, Figure 3.12. This suggests that CML progenitors may outgrow normal CD34$^+$ cells in cytokine limited conditions. In addition, the proliferation of CML CD34$^+$ cells was markedly enhanced in cultures with GM-CSF. This finding suggests that the CML progenitors are not totally cytokine independent. Other evidence to support this finding is in Chapter 7 in which the FLICA assay was used to examine effects on apoptosis. Primary CML CD34$^+$ cells did not maintain cell survival in culture in contrast with those with GM-CSF. Cultures had spontaneous progressive death over 3 days with the higher apoptotic index than the cells treated with GM-CSF. Together, these data suggest that there are additional proliferation and survival pathways which are not induced by BCR/ABL which can be stimulated by cytokines. Or alternatively, in primary CML CD34$^+$ cells, BCR/ABL activity is insufficient to saturate growth and survival pathways.

Imatinib, a 2-phenylaminopyrimidine derivative, which has become the first line therapy for chronic-phase CML, profoundly blocks the ATP binding site on the tyrosine kinase domain of the ABL portion of the CML fusion protein BCR/ABL, leading to the attenuation of downstream signalling activity, resulting in lowered proliferation and induction of apoptosis in CML cells(Buchdunger, *et al* 1995, Druker, *et al* 1996). Cytokines of the IL-3 family transduce similar set of signals as those stimulated by BCR/ABL(Steelman, *et al* 2004), although they do not fully overlap. To test that cytokines in this family are better able to stimulate CML CD34$^+$ cell proliferation, GM-CSF, IL-3 and the non-family member G-CSF were used to examine the enhancement of the spontaneous proliferation of CML CD34$^+$ cells using CFSE dye dilution technique (Chapter 3). It was found that G-CSF did not enhance cell division compared with the other two cytokines, suggesting that although CML CD34$^+$ express the G-CSF receptor, the stimulation of proliferation and survival through this receptor was not so strong.

Alternatively, the signaling through G-CSF receptors was not additive to the prominent activation of signals through BCR/ABL.

In the studies presented in this thesis, GM-CSF was used as the representative cytokine in its family. Primary CML $CD34^+$ cells, unlike the wt-BCR-ABL/CTLEN cell line, do not appear to have saturation of signal transduction pathways acted on by BCR/ABL, leaving unsaturated pathways able to be activated by cytokine receptor signalling. The wt-BCR-ABL/ CTLEN cell line has very active spontaneous proliferation which cannot be enhanced further by GM-CSF. When the kinase actibity of BCR/ABL was blocked by imatinib, primary CML $CD34^+$ cells could maintain proliferation by using a lower threshold of GM-CSF concentration than the BCR/ABL expressing cell line wt-BCR-ABL/CTLEN. The complete function of BCR/ABL may be associated with the higher level of BCR/ABL protein found in cell lines where the BCR/ABL is over-expressed or under a strong promoter. The high level of BCR/ABL does not relate to resistance to imatinib, which is usually due to BCR/ABL mutation, but does relate to the low response of cells to the cytokine. Therefore, the level of BCR/ABL could predict the cytokine independence. This was also previously shown in the TF-1/ wt-BCR-ABL cells in responses to EPO (erythropoietin) induction of cell growth (Uchida, *et al* 2004), and 32D/wt-BCR-ABL cells in response to GM-CSF (Keeshan, *et al* 2002). It was that EPO and GM-CSF only could not enhance the cell proliferation over spontaneous proliferation driven by BCR/ABL in TF1/wt-BCR-ABL and 32D/wt-BCR-ABL respectively. Imatinib inhibits the phosphorylation of BCR/ABL, and in addition, it may induce the activation of other signals which may be essential for cytokine activation as shown in previous studies (Jacquel, *et al* 2003). Therefore, inhibition of BCR/ABL by imatinib may induce cells to better respond to GM-CSF, because it imitates a low level of BCR/ABL.

In contrast to cell lines, the ability of GM-CSF to induce the proliferation of primary CML $CD34^+$ cells is in a concentration dependent manner. However, it reached a maximal level when the concentration of GM-CSF was equal or greater than 300 pg/mL. This suggested that to overcome the spontaneous proliferation driven by BCR/ABL needed threshold level of GM-CSF, and this should be happened to other cytokines. The proliferation of primary CML $CD34^+$ cells could be effectively inhibited by imatinib. However, their proliferation after inhibition could be reversed to the same level as their spontaneous proliferation, by

using exogenously applied GM-CSF (Chapter 3). The modulation of imatinib by GM-CSF on primary CML $CD34^+$ cells suggested BCR/ABL in primary CML did not have complete activation of all the signals which could still be stimulated by GM-CSF even the cultures contained imatinib.

The viability of cells examined by 7-AAD dye exclusion corresponded very well with the proliferation data. When normalizing the data of the parent cell recovery as shown in Chapter 4, most cells had the same responses to imatinib suggesting GM-CSF did not change the sensitivity of cells to imatinib, but only maintains cell survival. In the other hand imatinib still blocks the function of BCR/ABL in the culture with GM-CSF. GM-CSF could not override BCR/ABL inhibited by imatinib in terms of the blockade of the major subtrates activated by BCR/ABL such as Crkl, therefore GM-CSF does not necessarily change the sensitivity of cells to imatinib. However, the finding that GM-CSF can induce cell survival suggested that GM-CSF receptor interacts other signals which were not inhibited by imatinib. However, a patient whose CML $CD34^+$ cells showed good responses to imatinib showed that added GM-CSF could induce cells to partially resist imatinib. Therefore GM-CSF activity on these CML cells may replace key pathways stimulated by BCR/ABL.

The titration experiment of GM-CSF to examine the responses of CML $CD34^+$ cells to GM-CSF showed that GM-CSF needed a threshold concentration to enhance BCR/ABL activity as mentioned above (Chapters 3 and 4, about GM-CSF titration showing the similar pattern of responses). Therefore, by this reason, CML $CD34^+$ cells may not produce other cytokines, or produce levels insufficient to enhance the proliferation activity driven by BCR/ABL. In addition, the concept of a threshold level indicates that CML $CD34^+$ might not produce GM-CSF. This led us to examine the direct GM-CSF production by CML cells of both CML $CD34^+$ and non-$CD34^+$ cells using ELISpot. Interestingly, GM-CSF production was predominantly a property of non-$CD34^+$ CML cells, and very few CML $CD34^+$ cells were able to produce GM-CSF. The ability of non-$CD34^+$ CML cells to produce GM-CSF acting on CML $CD34^+$ cells was confirmed by the bystander experiments.

Using purified CML $CD34^+$ progenitors precultured with combination of GM-CSF and imatinib followed by assay of colony formation in semi-solid agar, we showed that GM-CSF allow the progenitor cells to survive levels of imatinib able to kill the majority of the progenitors. In addition, GM-CSF markedly preserved CML progenitor cells which had a significant level of viability decrease in serum free culture, compared with the cells without any treatment, confirming that primary CML $CD34^+$ cells are not completely cytokine independent.

8.3. *CML $CD34^+$ cells could survive in the presence of imatinib when co-cultured with non-$CD34^+$ CML 'bystander' cells.*

The contribution of non-$CD34^+$ cells to the response of CML $CD34^+$ to imatinib was first examined in this thesis. The bystander cell concept aimed to imitate the response of CML $CD34^+$ cells growing in the bone marrow environment containing other cells, to imatinib treatment. Imatinib could not suppress the cell proliferation of CML $CD34^+$ cells in mixed mononuclear cultures, but could strongly suppress proliferation of CML $CD34^+$ cultured alone. This suggests there were protective factors such as cytokines produced by the non-$CD34^+$ cells acting on CML $CD34^+$ cells. We initially expected that cytokines other than GM-CSF were produced in the environment containing non-$CD34^+$ cells. However, by using E21R, a GM-CSF α receptor specific blocking agent, the cell proliferation was completely suppressed to the level of the proliferation of imatnib-treated CML $CD34^+$ cells without bystander cells in cultures. This suggested that GM-CSF was the predominant cytokine in this system. Several attempts were made to identify if other cytokines such as IL-3 or G-CSF are involved in the bystander effect using other cytokine blocking agents, but it is very difficult to get adequate number of healthy non-$CD34^+$ and $CD34^+$ CML cells from cryopreserved samples. A further mechanism for the reduction of cell proliferation of CML $CD34^+$ cells in mixed cultures by E21R may come from E21R blocking GM-CSF receptor of putative GM-CSF dependent IL-3 producing cells. It would be instructive to use IL-3 α-receptor specific blocking agents to test whether or not the bystander cells contain such IL-3 dependent GM-CSF producing cells, because blockade of one of these cytokines will affect the production of the other cytokines and thus enhance the blockade of proliferation of CML $CD34^+$ cells. We know from Chapter 3 results that CML $CD34^+$

cells definitely contained the receptors of IL-3, GM-CSF and G-CSF. Therefore, if the bystander cells produce both IL-3 and GM-CSF at sufficient levels, the proliferation activity of cells should be greater than the cells with GM-CSF alone, but the results did not show that expected activity. This is because the proliferation activity induced by the bystander was completely blocked by E21R. Therefore, if a single cytokine blocking agent can completely inhibit the bystander effect and we believe cells could produce threshold levels of other cytokines apart from GM-CSF able to act on CML $CD34^+$ cells, those cells contained in the bystander could be IL-3 dependent GM-CSF producing cells or GM-CSF dependent IL-3 producing cells. If sufficient CML $CD34^+$ cells and the specific blocking agents were available, these possibilities could be examined.

8.4. *The wt-BCR-ABL/CTLEN showed that GM-CSF receptor could not enhance the effect of BCR/ABL unless BCR/ABL kinase activity was completely blocked.*

The wt-BCR-ABL/CTLEN cell line which is transfected with human BCR/ABL and the complete human GM-CSF receptor was used as an *in vitro* model to examine the signaling through both BCR/ABL and GM-CSF receptor. As this is a renewable cell line, the large number of cells necessary to examine signaling pathways by Western Blot could be obtained. In contrast, primary CML $CD34^+$ cells could not be obtained in sufficient numbers to do these experiments. The finding with the wt-BCR-ABL/CTLEN cells is different from the primary CML $CD34^+$ cells in terms of the effect of GM-CSF enhancement of spontaneous proliferation, which has been mentioned above. However, the pattern of the proliferation of CTLEN cells is quite similar to the CML $CD34^+$ cells in bystander cultures in which the added GM-CSF did not change the pattern of the cell spontaneous proliferation. This suggests that GM-CSF or other cytokines may activate other signals which are not involved in BCR/ABL signaling in the primary CML $CD34^+$ cells to get full proliferation, but the BCR/ABL in CTLEN could function to signal more completely than in the primary CML $CD34^+$ cells. We still believe that although CTLEN could provide complete function of BCR/ABL, there still some 'spare' signals left for the activation by GM-CSF receptor signaling. Titration of the GM-CSF effect on the proliferation of cells by MTS assay, demonstrated that GM-CSF maintains the proliferation of cells to the same level of the spontaneous proliferation of the wt-BCR-ABL/CTLEN at the concentration greater than 10ng/mL, which is much higher than its maintenance

concentration for primary CML cells. This finding suggested that the response to GM-CSF may be dependent on the level of BCR/ABL production in cells. In turn, the complete function of BCR/ABL may be dependent on the level of BCR/ABL as well. With this concentration of GM-CSF, CTLEN could escape imatinib until a toxic concentration of Imatinib was reached. This indicated that GM-CSF could replace the function of BCR/ABL to maintain cell survival, but the identification of signals involved requires elucidation.

8.5. *Signals associated with GM-CSF modulation of imatinib effect on CML $CD34^+$ cells*

Phospho-Akt is a protein involved in Bad and Bim activation. The activated Akt or phosphorylated Akt (p-Akt) was not be decreased in the wt-BCR-ABL/CTLEN in culture with imatinib, unless the concentration of imatinib reaches toxic levels. The level of expression of p-AKT is not particularly high, it was sufficient to bind with other proteins such as Bad and Bim, caising their phosphorylation as shown in Chapter 6, using an Akt immunoprecipitation approach. Only phosphorylated Bad and Bim bind to Akt, and the level of binding was reduced in culture with imatinib, which is consistent with the MTS cell proliferation assay findings. Addition of GM-CSF resulted in enhanced binding of phosphorylated Bim and Bad. Total Bim and Bad protein was increased with 10 μM imatinib, or alternatively increased p-Akt was able to bind more of these proteins. At 10 μM imatinib may stimulate c-Jun n-terminal kinase through the DNA damage, which will disrupt the binding of 14-3-3 and p-Akt, and thus increase the binding of Bim and Bad to p-Akt and maintain the level of 14-3-3 binding(Tsuruta, *et al* 2004). This has been confirmed in Chapter 6 and a similar mechanism was found. 14-3-3 may prevent p-Akt from being destroyed. If 14-3-3 binding is disrupted by high dose imatinib, it becomes active and binds to other substrates and may therefore allow cell survival without proliferation.

In reciprocal experiments, Bim was immunoprecipitated and found to have increased binding with p-Akt with increased concentration of imatinib which supported the concept above (Chapter 6, Figure 6.9) (Qi, *et al* 2006). Interestingly, Bim had a very low binding with p-Erk, another protein activating Bim phosphorylation, but this binding became

stronger in response to GM-CSF (Chapter 6, Figure 6.9). This reflected increased cell survival, presumably because of greater Bim phosphorylation. These activated substrate mechanisms induce cells to have a potential to survive imatinib and to have cell activity to proliferate immediately after stimulation. The important signal here is p-Akt, as its level was not decreased even at high concentrations of imatinib and it may be the key mechanism of cell insensitivity to imatinib.

Bim levels are regulated by proteosome degradation as well as production, which is controlled by cytokine deprivation. We have already shown here that Bim level was increased by the inhibition of BCR/ABL by imatinib. Interesting, imatinib at higher dose could inhibit the degradation as well, in both in the cultures with or without GM-CSF. It would be an interesting further study if imatinib could block the proteosome degradation pathway.

Phospho-Erk, a protein in the MAPK family, was found to have constitutive phosphorylation, although the level of phosphorylation was not very high. The level of phospho-Erk is reduced as the concentration of imatinib increased. Interestingly, the level is significantly increased in response to GM-CSF, and is also higher still in the cultures with higher concentrations of imatinib plus GM-CSF. This also correlates with the finding on the binding of p-Erk to Bim when Bim was immunoprecipitated. P-Erk may be an example of signals which are suppressed in this cell line by the dominant activation of other substrates by BCR/ABL. However, it appears to be a spare signal which could still be stimulated by GM-CSF receptor in spite of imatinib blockade of BCR/ABL function.

Bim was found to bind with BCL-XL and BAX, which have opposing functions in terms of apoptosis: BCL-XL is an anti-apoptotic protein and BAX is a pro-apoptotic protein. BCL-XL was increased which is compatible with the increased Bim in the cultures with imatinib, and then its level is decreased with addition of GM-CSF. However, BCL-XL levels is correlated with STAT5 activation from the previous report because phospho-STAT5 has a direct transcribed of BCl-XL (Horita, *et al* 2000, Sillaber, *et al* 2000). That finding the level of BCL-XL binding to Bim becomes increased may be explained by the total BCL-XL level being increased by GM-CSF, which in turns activates p-STAT5 and BCL-XL production. This may increase BCL-XL binding with Bim as well. A decreased

binding of Bax to Bim was not apparent at the short time incubation with GM-CSF compared with the cells without GM-CSF, but it became substantial with longer incubation. Together, these findings supported the concept that GM-CSF inhibits the proapototic signals induced by imatinib because Bim activates Bax and inhibits BCl-XL . The binding of Bim to these proteins then induces apoptosis.

8.6. *Comparison between primary CML CD34$^+$ cells and the wt-BCR-ABL/CTLEN cell line.*

The time-course experiment of primary CML CD34$^+$ CML cells provided some interesting information about the bystander effect of non-CD34$^+$ cells acting on CML CD34$^+$cells Whilst the bystander cells kept cell proliferation as high as the cells with the exogenously applied GM-CSF, E21R could reduce this effect down to the level of the control suggesting GM-CSF was still the predominant cytokine in the bystander system even though cells were cultured for 5 days. From this stage, CML CD34$^+$ cells are not completely the cytokine-independent and GM-CSF was identified as a key cytokine to investigate the preserved signals downstream after inhibition of BCR/ABL.

A patient whose CML CD34$^+$ cells was proven before to have a good response to both exogenous applied GM-CSF and bystander cells showed Western Blot results somewhat different to the wt-BCR-ABL/CTLEN cell line. Both cell types also showed GM-CSF could not induce Crkl phosphorylation, one of the major substrates signals of BCR/ABL signalling. Crkl is an adaptor protein which links to other substrates downstream, including PI3-K, and potentially the Ras/Raf/MAPK pathway. In this patient cells, p-Akt, a substrate belonging to the PI3-K/Akt pathway, was found to be a little increased in response to GM-CSF, which is similar to the profile seen in the cell line. However, when investigating other proteins associated with p-Akt activation such as Bim, it was found that the Bim level was decreased after GM-CSF exposure, but had a higher proportion in the phosphorylated form, suggesting p-Akt may induce Bim phosphorylation. P-Erk was not reduced in cultures with imatinib and its level was still maintain in the cultures with imatinib plus GM-CSF, while the level of Bim phosphorylation was changed with imatinib treatment, suggesting that p-Erk may not play role in activation of Bim in the primary cells. The expression of p-Erk in the presence of imatinib suggested that primary CML cells may

have some proliferation activity, even at high concentrations of imatinib. Thus regulation of total cell number after culture may mainly be from the regulation of apoptosis, as the activity of Bim was seen to be changed with imatinib. Although the expression of p-Erk in the primary cells is different from CTLEN cells, cell survival will still be controlled by p-Akt and Bim. P-Akt may be an important route for the cytokines such as GM-CSF to promote cell survival in the presence of imatinib.

8.7. *The cross talk between BCR/ABL and activated GM-CSF signalling in CML $CD34^+$ cells*

Both GM-CSF and BCR/ABL could activate overlapping signalling substrates. However, as described above, BCR/ABL may predominately stimulate the signalling and reduce the apparent effect of GM-CSF receptor signalling. BCR/ABL inhibits apoptosis by the activation of signalling through PI3-K which activates the phosphorylation of Akt and m-TOR. The p-Akt is then further activated by Protein C kinase. The activated Akt binds to and phosphorylates Bim and Bad. Phospho-Bim and phospho-Bad are sequestered by 14-3-3 which prevent the binding to other proteins, such as prevention of Bim binding with BCL-XL and Bax to inhibit apoptosis. The level of 14-3-3 that is not reduced when Akt was immunoprecipitated may be from secondary association such as 14-3-3 with Bim or with Bad.

Otherwise, 14-3-3 may bind to p-Akt to protect the active p-Akt from destruction and help to bring other proteins to associate with p-Akt and become phosphorylated. The 14-3-3 binding to Bim is not reduced by imatinib, which is associated with the phosphorylated Bim level. This is confirmed by examining p-Akt on the Bim-immunoprecipitated membrane. The level of p-Akt was not reduced suggesting the phosphorylation of Bim should still be retained.

In BCR/ABL cells, Bim was reduced by the action of BCR/ABL to reduce the production of Bim through p-Akt activation which further activate FOXO to be phosphorylated form. FOXO, a transcription factor, could activate Bim production by migrating to nucleus. The phosphorylated form of FOXO will be inhibited by 14-3-3 to prevent the nulear migration of p-FOXO and then inhibit Bim production (Qi, *et al* 2006).

The m-Tor and p-Akt both are directly activated by PI3-K, but only p-Akt can phosphorylate Bim (Qi, *et al* 2006). Phospho-Bim is sequestered by 14-3-3, preventing the association of p-Bim to Bad and BCL-XL and then prevent cells from apoptosis. The m-Tor migrates to nucleus and inhibits the production of Bim. This mechanism will therefore inhibit apoptosis.

Another mechanism which regulates Bim level is the proteosome degradation pathway(Qi, *et al* 2006) which can be activated by BCR/ABL (Figure 6.5 and 7.4). Both Bim and phospho-Bim level will be reduced by the actvation of this pathway.

Upon exposure to imatinib, mechanisms will be altered as shown in the diagram in Figure 6.11, except the proteosome degradation pathway which has not been reported previously to be affected by imatinib. The findings from both the primary CML cells and wt-BCR-ABL/CTLEN cell lines are that at high concentrations of imatinib, both the level of Bim and phosphorylated Bim was increased, suggesting imatinib could inhibit proteosome degradation. This effect is general because it also increased all the isoforms of Bim when using imatinib with GM-CSF, not only when imatinib was used alone. The clinical implication is that the inhibition of proteosome degradation by imatinib may potentially enhance the effect of cytokines such as GM-CSF. Modurating activation of proteosome degradation may be another strategy to enhance the effect of imatinib.

8.8. ***Summary***

CML cells can grow spontaneously in SDM without exogenous cytokines. However, the behavior of BCR/ABL cell lines and the primary CML cells are different in part because the level of activity of BCR/ABL is different. How complete the BCR/ABL function is, depends on the level of BCR/ABL in the cells, but not the percent BCR/ABL positive cells by FISH. The primary CML $CD34^+$ cells in this thesis were found to be not completely cytokine independent and needed additional signal activation to complement the function of BCR/ABL compared with cell lines. Although, the BCR/ABL activity in the wt- BCR-ABL/CTLEN cells could not activate all the major signals, they still have some remaining signalling pathways which can be activated by GM-CSF, including p-Erk1/2 and p-Akt. Bim, the pro-apoptotic protein activated by p-Erk and P-Akt, was found to be increased in

the presence of imatimib and decreased in the presence of GM-CSF, which correlated with cell apoptosis and cell survival. Interestingly, Bim and p-Bim were found to be increased at higher concentration of imatinib both with and without GM-CSF suggesting imatinib might inhibit proteosome degradation. It is worth furthering investigation into how imatinib interacts with the proteosome degradation pathway. In short, BCR/ABL activation of some substrates may suppress the expression of other substrates, and its activation may not induce all the important pathways relating to cell proliferation and survival. These substrates or pathways can still be activated by cytokines, such as GM-CSF, and maintain CML cell survival when BCR/ABL is blocked by imatinib.

The findings here show that the mechanism of cytokine and BCR/ABL signalling pathway is complex. These results suggest that cytokines using the β common chain of the IL-3 family may potentially protect CML progenitors during therapy with tyrosine kinase imatinib, and identifies additional target which may be useful in enhancing CML treatment.

Chapter 9.

Bibliographies

9. Bibliographies

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