Investigation of the role of autocrine and paracrine growth factors in the survival and proliferation of chronic myeloid leukaemia stem and progenitor cells

by

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Abstract

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder arising in a haemopoietic stem cell (HSC) as a result of the reciprocal translocation between the long arms of chromosomes 9 and 22 (t9;22), leading to the formation of the fusion oncogene *BCR-ABL*. BCR-ABL has constitutive tyrosine kinase (TK) activity which drives, at least during the chronic phase (CP) of the disease, myeloid progenitor cells expansion through terminally differentiated cells and is necessary for the transformed phenotype. The introduction at the end of the last century of BCR-ABL TK inhibitors (TKI) has dramatically changed the management of newly diagnosed CP CML patients as the vast majority achieve deep molecular responses while enjoying good quality of life when treated with TKI. However about 20% of patients still show various degree of resistance to all currently available TKI while in those achieving deep responses, there is compelling evidence of persistent minimal residual disease demanding lifelong treatment which has obvious implications in terms of compliance, adverse events and costs.

It is now known that the main reason for disease persistence in CML patients treated with TKI lies in the insensitivity of the most primitive CML leukaemia stem cell (LSC). More recent evidence has demonstrated that, in contrast to more mature leukaemic progenitor cells, CML LSC are not addicted to BCR-ABL kinase activity but rather rely on other stem cell intrinsic pathways for their survival. The main focus in the CML field is therefore to identify these pathways while also trying to exploit them therapeutically to achieve CML LSC eradication and as a result disease cure.

Growth factor (GF) signals are known to provide survival cues to CML stem and progenitor cells (SPC) and potentially support their survival even in the presence of TKI. Moreover CML SPC are also known to produce higher levels of some GFs via an autocrine loop and support their survival and proliferation through this mechanism. In this thesis, the characterisation of the autocrine GF production by CML SPC was extended while also investigating the role of several GFs and downstream signals in survival, proliferation and self-renewal of CML SPC. Whenever possible, the consequences of therapeutic targeting of these signals on CML SPC survival and proliferation were also assessed *in vitro*.

In particular the role of the intracellular janus kinase (JAK) 2, which relays several myeloid GF signals, such as those from interleukin (IL)-3 and granulocyte macrophage colony-stimulating factor (GM-CSF), in CML SPC survival and proliferation was

investigated mainly because higher levels of autocrine expression of GM-CSF by CML SPC relative to normal were demonstrated, while autocrine IL-3 production by CML SPC had already been shown. Moreover the cognate receptor of both GM-CSF and IL-3 (CSF2RB) was also shown to be expressed at higher levels in CML SPC relative to their normal counterparts, further supporting investigations on the role of JAK2 in CML SPC biology. Indeed targeting JAK2 with small molecule inhibitors in CML SPC *in vitro*, particularly in the presence of maximal BCR-ABL TK inhibition, resulted in increased apoptosis, reduced proliferation and colony output of CML SPC. The JAK2 inhibitor plus TKI combination treatment, compared to either single agent, further reduced survival of the more primitive quiescent LSCs *in vitro,* while also reducing engraftment of primary CML $CD34^+$ cells *in vivo* in immunocompromised hosts. Although a degree of toxicity to normal haemopoietic stem and progenitor cells (HSPC) was observed, this was not as great as seen in CML SPC, thus suggesting that a therapeutic window for using JAK2 inhibitors in CML patients might be present when a carefully selected concentration of these compounds is chosen.

Tumour necrosis factor (TNF)-α was another GF shown to be produced in an autocrine fashion at higher levels by CML SPC relative to normal HSPC. Moreover its levels of production by CML SPC were not modulated by BCR-ABL TK activity. Using a small molecule TNF-α inhibitor and exogenous TNF-α, it was shown that autocrine TNF-α acts as a survival and proliferative signal in CML SPC. Moreover its role became even more important in the presence of TKI, as combining TNF-α inhibition with TKI led to high levels of apoptosis in CML $CD34^+$ cells, including the more primitive quiescent population, while also causing increased apoptosis in a population enriched for CML LSCs based on its surface marker expression ($CD34^+$ $CD38^-$).

Finally given the known importance of quiescence and self-renewal pathways in CML LSC persistence following TKI treatment, the role of transforming growth factor (TGF)-β1 and novel neurotransmitter mediated pathways in CML LSC quiescence and self-renewal was investigated based on the findings of a genome and epigenome-wide screen of primary CML LSCs and normal HSCs carried out in our laboratory. Using *in vitro* assays the putative role of the neuromediators norepinephrine and acethylcoline in CML LSC self-renewal was demonstrated. Moreover the role of TGF-β1 in inducing primary CML LSC quiescence mainly by modulating the AKT pathway was also demonstrated.

Overall the work presented in this thesis has furthered our understanding of the role of both autocrine and paracrine known and novel regulators of haemopoiesis in several aspects of CML SPC biology such as their survival, proliferation and self-renewal. Furthermore the efficacy in eradicating CML SPC of therapeutic strategies targeting some of these GF signals has been explored *in vitro,* thus providing evidence supporting their subsequent testing in *in vivo* assays and in due course in clinical studies. It is hoped therefore that the work presented will contribute to devise novel therapeutic strategies to eradicate CML LSC and in turn lead to a cure for CML patients.

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Related publications

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Dedication

This thesis is dedicated to my father, Martino, a constant source of inspiration. I am sure that from afar he is still taking pleasure in all my achievements.

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Author's Declaration

I declare that all data presented in this thesis is my own work, unless otherwise stated.

Definitions/Abbreviations

5HT	Serotonin
7AAD	7 aminoactinomycin D
ABC	ATP-binding cassette
ACH	Acethylcoline
ADRA2A	α2 adrenergic receptor
ALBA	Human albumin solution
alloBMT	Allogeneic bone marrow transplantation
AML	Acute myeloid leukaemia
AP-1	Activator protein-1
APC	Allophycocyanin
APO-E	Apolipoprotein-E
ATP	Adenosine triphosphate
BC	Blast crisis
BCA	Bicinchoninic acid
BCR-ABL	Breakpoint cluster region-abelson
BFU-E	Burst forming unit – erythroid
BIT	Bovine serum albumin/insulin/transferrin
BM	Bone marrow
BOS	Bosutinib
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCyR	Complete cytogenetic response
cDNA	Complementary deoxyribonucleic acid
CFC	Colony forming cell
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU-E	Colony forming unit – erythroid
CFU-GM	Colony forming unit – granulocyte and macrophage
CHR	Complete haematological response
CHRM3	Muscarinic receptor 3
CKI	Cyclin dependent kinase inhibitor
CLP	Common lymphoid progenitor
C_{max}	Maximal concentration
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
CMR	Complete molecular response
CP	Chronic phase
CSC	Cancer stem cell
CSF	Colony-stimulating factor
DA	Dasatinib
DAG	Diacyglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNASE	Deoxyribonuclease
dNTPs	Deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridistaion
FITC	Fluorescein isothiocyanate
FLT-3L	Fms-like tyrosine kinase receptor-3 ligand
FOXO	Forkhead box subgroup O
FSC	Forward angle side-scatter
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GF	Growth factor
GFP	Green fluorescent protein
GLU	Glutamate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
GTP	Guanosine Triphosphate
HBSS	Hank's buffered salt solution
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor
HiGF	High growth factor
HIS	Histamine
HPC	Haemopoietic progenitor cell
HSC	Haemopoietic stem cell
HSPC	Haemopoietic stem and progenitor cells
IAP	Inhibitor of apoptosis
IC_{50}	50% inhibitor concentration
IFC	Integrated fluidic circuit
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-4Rα	Interleukin-4 receptor alpha
IM	Imatinib
IMDM	Iscove's Modified Dulbecco's Medium
INC	INCB18424
IP3	Inositol 1,4,5-trisphosphate
iTRAQ	Isobaric tags for relative and absolute quantitation
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JAK	Janus kinase
KCl	Potassium chloride
KO	Knockout

LIF	Leukaemia inhibitory factor
LPC	Leukaemia progenitor cells
LSC	Leukaemia stem cells
LTC-IC	Long-term culture–initiating cells
LT-HSC	Long term haemopoietic stem cell
LY	LY364947
MAP	Mitogen activated protein
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MEK	Mitogen extracellular kinase
MEM-α	Minimum Essential Medium-α
MFI	Mean fluorescence intensity
$MgCl_2$	Magnesium chloride
MIP-1α	Macrophage inflammatory protein-1
MMR	Major molecular response
MNC	Mononuclear cells
MPP	Multipotent progenitors
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTA	Materials transfer agreement
NaCl	Sodium chloride
NEEA	Non-essential aminoacids solution
NEPI	Norepinephrine
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NL	Nilotinib
NOD/SCID	Non obese diabetic/severe combined immunodeficient
NSG	NOD/SCID-interleukin 2 receptor γ-chain-deficient
OS	Overall survival
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PCyR	Partial cytogenetic response
PDGFR	Platelet derived growth factor receptor
PDK	Phosphoinositide dependent kinase
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PGF	Physiological growth factor
Ph	Philadelphia
PIP2	Phosphatidylinositol -4,5- bisphosphate
PIP3	Phosphatidylinositol -3,4,5- bisphosphate
PLCB1	Phospholipase C β1
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukaemia
PON	Ponatinib
PRKC	Protein kinase C
PRKCE	Protein kinase C epsilon

PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time-polymerase chain reaction
RAS	Rat sarcoma
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RQ	Relative quantification
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Stem cell factor
SDF1	Stromal cell derived factor 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFM	Serum free medium
SH	SRC homology
SIRT1	Sirtuin 1
SPC	Stem and progenitor cells
SRC	Sarcoma virus gene
SSC	Side angle-light scatter
STAT	Signal transducer and activator of transcription
ST-HSC	Short term haemopoietic stem cell
TG	TG101209
TGF	Transforming growth factor
TGF-β1 R1	TGF-β1 receptor 1
TGF-β1 R2	TGF-β1 receptor 2
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TNFR1	Tumour necrosis factor alpha receptor 1
TNFR2	Tumour necrosis factor alpha receptor 2
TNF	Tumour necrosis factor
TNF-α inh	Tumour necrosis factor alpha inhibitor
TPO	Thrombopoietin
TRIS	Tris (hydroxymethyl)aminomethane hydrochloride
UT	Untreated
VEGF	Vascular endotelial growth factor

1 Introduction

1.1 Normal haemopoiesis

1.1.1 The haemopoietic stem cell (HSC)

Haemopoiesis is the process leading to the formation of all different types of blood cells starting from a HSC, normally residing in the bone marrow (BM). Because of the very short life span of most mature blood cells, haemopoiesis is an ongoing process producing between 10^{11} to 10^{12} blood cells every day in an adult human at steady state[1]. This process is made possible by the two main properties of HSC[2]:

1) multipotency, defined as the ability to generate differentiated committed progenitor progeny through asymmetrical cell division which in turn gives rise to all mature blood cells;

2) long-term self-renewal, defined as the lifelong ability to generate a daughter cell identical to the parent through symmetrical cell division, thus maintaining an overall constant pool of HSCs during the lifespan of an organism

HSCs are the only haemopoietic cells displaying these two properties. Committed progenitors retain proliferative and developmental capacity although this is progressively lost as they mature from multipotent through oligopotent and lineage restricted progenitor cells into terminally differentiated cells which are unable to grow further[1].

The first evidence supporting the existence of HSC was the discovery in mice of a population of clonogenic BM cells able to generate myeloerythroid colonies in the spleen of lethally irradiated hosts[3]. Moreover these colonies contained clonogenic cells that could be transferred to secondary lethally irradiated hosts and reconstitute all blood cell lineages, thus proving their self-renewal potential[4]. Subsequently it was found that a high proportion of cells of the lymphoid system belonged to these same clones[5]. These colony forming cells therefore showed capacity for proliferation, differentiation into all lineages and self-renewal and were proposed to contain HSC. Subsequently the differential capacity of reconstituting haemopoiesis in lethally irradiated hosts from mouse BM cells prospectively isolated on the basis of their cell surface marker expression by multiparameter fluorescence activated cell sorting (FACS) has enabled the purification of murine HSC with a purity of 1 in 2^6. Using the same functional and phenotypic assays it has also been possible to define

the ontogeny of all mature blood cells starting from the HSC. HSCs can be divided into long-term (LT)-HSCs and short-term (ST)-HSCs based respectively on their long-term or more limited (about 8 weeks) self-renewal capacity[7]. ST-HSC give rise to the multipotent progenitors (MPP) only capable of very limited self-renewal[8]. MPP then produce either of the two main lineage restricted progenitors, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). These are functionally committed progenitors which have taken an irreversible maturation step towards the lymphoid[9] or myeloid lineage[10]. From them through progressive maturation steps the mature differentiated blood cells are eventually produced (figure 1-1).

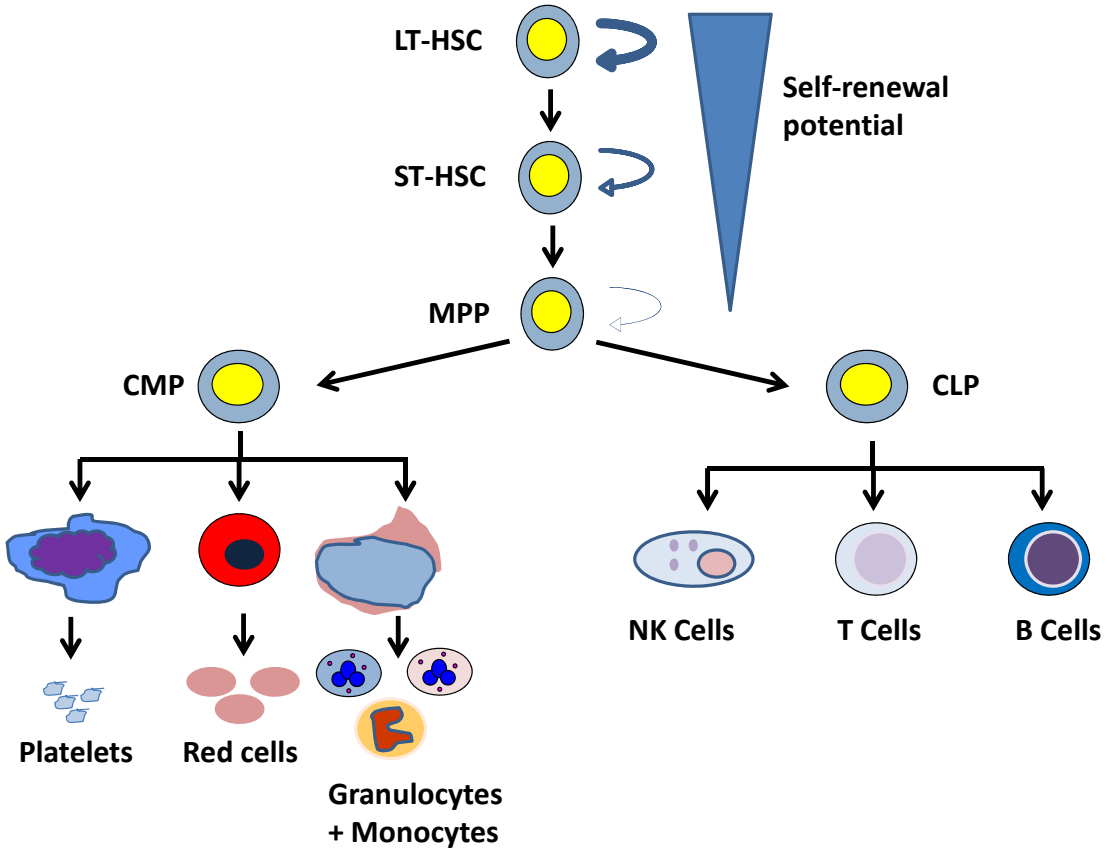


Figure 1-1 Ontogeny of mature blood cells
Blood cells are produced starting from a LT-HSC which has maximal self-renewal potential. ST-HSCs have only short lived self-renewal potential while MPP have almost negligible self-renewal potential. From MPP lineage committed progenitors are derived (CMP and CLP) and from them through other more differentiated intermediate progenitors (not shown) the terminally differentiated cells. Abbreviations (only those not explained in main text): NK, natural killer.

Prospective isolation of human HSC has been lagging behind the murine counterpart, mainly because of suboptimal functional assays. While in mice competitive *in vivo* repopulating assays can be used to demonstrate sustained self-renewing and multipotent differentiation capacity of HSC, the same cannot be performed in humans for obvious ethical reasons. Therefore two main surrogate functional assays have been employed to

identify human HSC. In the long-term culture-initiating cell assay (LTC-IC), candidate BM haemopoietic cells are cultured for 6 weeks or longer *in vitro* on an adherent layer of BM feeder cells in an attempt to mimic the BM microenvironment. Thereafter cells are transferred into semi-solid medium containing growth factors (GFs) and their ability to generate colonies is measured. The ability of candidate cells to produce differentiated colonies following long-term culture (>6 weeks) is considered evidence of self-renewal capacity and multilineage differentiation potential. As a result the LTC-IC assay is thought to identify primitive progenitors and HSC[11]. Alternatively the ability of prospectively isolated subsets of human haemopoietic stem and progenitor cells (HSPC) to reconstitute long-term multipotent human haemopoiesis in *in vivo* xenotransplantation assays using immunodeficient mice has been used to identify and quantitate human HSC[12,13]. Despite their limitations, thanks to these assays a progressive and more refined characterisation of human HSC has been achieved.

The main positive (and first to be described) surface marker for human HSC is the transmembrane glycoprotein CD34[14]. Its biological function in haemopoiesis is poorly understood and in fact could not even be relevant because despite the reduced *in vitro* colony forming activity of their haemopoietic progenitors, CD34 deficient mice have normal peripheral blood counts and respond to haemopoietic stress as well as wild-type mice[15]. CD34 is expressed in 1 to 2% of BM mononuclear cells (MNC) and its levels of expression correlate with *in vitro* colony formation and LTC-IC activity with highest expression levels in the earliest haemopoietic cells[11,14,16]. Despite this, even cells expressing high levels of CD34 ($CD34^{high}$) are still a heterogeneous population, including both HSCs and more committed haemopoietic progenitor cells (HPCs). Subsequently it was shown that only a small fraction (about 1 to 10%) of the $CD34^{high}$ population, which does not express mature lineage markers (Lin^-), and has low or undetectable levels of expression of the surface marker CD38 (another poorly defined transmembrane protein[17]), contains cells with both lymphoid and myeloid differentiation potential[18-21], with a frequency of 1 in 617 in *in vivo* assays. Finally, more recently a further phenotypic characterisation of normal human HSC has been added based on expression of three other surface markers CD90, CD49f and CD45RA: therefore according to the most recent evidence the HSC activity can be isolated in as few as 1 in 10 purified $Lin^- CD34^+ CD38^- CD90^+ CD49f^+ CD45RA^-$ cells[22,23]. A summary of the current markers to identify human HSC and committed progenitors at various stages of differentiation is shown in table 1-1.

CELL TYPE	SURFACE MARKER PHENOTYPE
HSC	Lin^- High $CD34^+$ $CD38^-$ $CD90^+$ $CD49f^+$ $CD45RA^-$
MPP	Lin^- High $CD34^+$ $CD38^-$ $CD90^-$ $CD49f^-$ $CD45RA^-$
Lineage committed progenitors	$Lin^{+/-}$ $CD34^+$ $CD38^{+/-}$ $CD90^-$ $CD45RA^+$
Mature cells	Lin^+ $CD34^-$

Table 1-1 Surface immunophenotype of human haemopoietic cells at different stages of their development

The ability to isolate highly purified stem and progenitor cells (SPC) populations has also allowed their functional and molecular characterisation. It is now known that at steady state the LT-HSCs are mainly quiescent, i.e. in G0 phase of cell cycle. However they are still able to enter cell cycle with one report showing that up to 99% of LT-HSCs divide at least once every 57 days in mouse[24]. Conversely as LT-HSCs differentiate, a progressive reduction in the percentage of G0 cells is seen with a corresponding increase in the proliferation rate of each progenitor population[25]. Quiescence appears to promote their long-term haemopoietic reconstitution ability as the capacity of LT-HSCs to functionally engraft lethally irradiated hosts in competitive reconstitution assays rapidly decreases as they enter G1[25,26]. These results in mice have been paralleled by similar observations in human showing that G0 HSCs have higher engraftment potential and indeed maintaining quiescence both *in vitro* and *in vivo* resulted in improved repopulating capacity of human

HSC[27,28]. Taken together these results suggest that quiescence is a main regulator of the maintenance of a pool of functional LT-HSCs able to both self-renew and differentiate. The molecular regulators of the behaviour of HSPC are also now better understood and it has become clear that LT-HSC express higher levels of the negative cell cycle regulators, cyclin dependent kinase inhibitors (CKIs), such as *Cdkn1a/p21*, *Cdkn2a/p16* and *Cdkn1c/p57*. Conversely the expression of *cyclin D1*, a positive regulator of cell cycle, increases during differentiation[25].

1.1.2 Extrinsic regulators of normal haemopoiesis: cytokines, GFs and small molecules

Haemopoiesis requires fine regulation so that an adequate production of mature blood cells is provided at both steady state and during physiological stresses (such as bleeding and infection), while a constant pool of LT-HSC is maintained. This regulatory process is largely accomplished by the production of a family of extracellular soluble proteins collectively called GFs or cytokines (the two terms will be used interchangeably), which determine various biological responses by binding to surface receptors on different haemopoietic cell types. Cytokines play a crucial role in haemopoietic homeostasis as they regulate HSPC quiescence, proliferation, lineage commitment and differentiation, survival and apoptosis. They comprise proteins such as the interleukins (ILs), colony-stimulating factors (CSFs), interferons (IFNs), erythropoietin (EPO), thrombopoietin (TPO), transforming growth factors (TGFs), tumour necrosis factors (TNFs) and many others[29]. Other regulators of HSPC self-renewal have also been described which include chemokines such as stromal cell derived factor 1 (SDF1) and novel small molecules and proteins, such as the ligands of the embryonic morphogenic pathways Hedgehog (Hh), Notch and Wnt/β-catenin or, even more recently, several neurotransmitters[30,31]. Although these latter molecules are not usually considered part of the GFs and cytokine family, given their role in regulating haemopoiesis, they will be also presented and their function discussed in this section.

Most cytokines are produced locally within the BM niche where HSPCs normally reside, by stromal cells, fibroblasts, osteoblasts, adipocytes, endothelial cells and mature blood cells such as lymphocytes and macrophages[31-33]. However for some GFs, such as TNF-α or IL-8, low levels of autocrine production by HSPCs has been described[34], while in other cases a systemic endocrine production is also present (EPO by the kidney, TPO by the

liver)[33]. Moreover their production is also influenced by external stimuli, such as other cytokines, inflammation or hypoxia, all pertinent within the BM niche.

Each GF has a main action on the haemopoietic system based on its specific ability to support the formation of blood cells of multiple and specific lineages. So for example while granulocyte macrophage (GM)-CSF supports the survival and proliferation of earlier progenitors of both granulocytes and macrophages, EPO is the main regulator of erythrocytes, TPO of platelets and granulocyte (G)-CSF of neutrophils production[29]. This has led to the hierarchical classification of cytokines which mirrors the hierarchical haemopoietic cell development (figure 1-2). Although this classification is helpful in the overall analysis of the effects of various cytokines in haemopoiesis, it should be borne in mind that both redundancy and pleiotropism are two features of GF signalling. Redundancy determines that different GFs can exert similar or overlapping effects possibly as a consequence of them sharing common receptors and signalling pathways. Pleiotropism results in the ability of each cytokine to elicit different responses depending on several factors, including the cell context where they act, their concentration and presence of other signals[35,36].

Two models for the effects of cytokines in haemopoietic stem cell maintenance, differentiation and proliferation have been proposed. One model, called the stochastic model, states that lineage commitment and differentiation of HSPC is already determined within each haemopoietic cell by a transcriptional programme intrinsic to each cell, with the cytokines only providing permissive growth/survival signals. The alternative model, named the instructive model, states that GFs direct the lineage commitment and differentiation of multipotent haemopoietic cells and therefore have an instructive role in haemopoiesis[29,33]. Both models have been supported by experimental evidence. For example transgenic mice expressing human G-CSF receptor ubiquitously were able to support multilineage development of primitive MPP, both *in vitro* and *in vivo,* when stimulated with human G-CSF without inducing exclusive commitment to the myeloid lineage[37]. Conversely Kondo *et al* demonstrated that a CLP can be redirected to the myeloid lineage by stimulation through exogenously expressed IL-2 and GM-CSF receptors. Moreover by mutational analysis of the IL-2 receptor, they were also able to show that the signalling pathways respectively responsible for granulocyte and monocyte differentiation are separable[38]. It is possible that both models are true and that cytokine signalling can indeed regulate lineage commitment decisions, at least for some cytokines and at certain points during haemopoietic development. While however the survival and

growth signals triggered by the GFs are well described, the signals leading to lineage decisions are currently not yet clearly known. The high number of soluble regulators of haemopoiesis (currently estimated at over 80) and the complexity of their regulatory networks inevitably demands a degree of simplification when approaching this subject. As the main aim of the work presented in this thesis is to investigate the role of some of these regulators in normal and chronic myeloid leukaemia (CML) stem cell fate, a more in depth introduction to only the regulators involved in HSPC and myeloid cell development will be presented, particularly focusing on those that have been the subject of investigations in the work presented in this thesis. They will be presented in three different sections; the first concentrating on those GFs relevant to HSPC proliferation and myeloid differentiation (IL-3, GM-CSF, G-CSF); the second addressing some putative regulators of HSC maintenance (note the term maintenance is used here to signify preservation of self-renewal potential) and quiescence i.e. TGF-β1 and neurotransmitters; and the third focusing specifically on a truly pleiotropic cytokine TNF-α known for its varied effects depending on cell context.

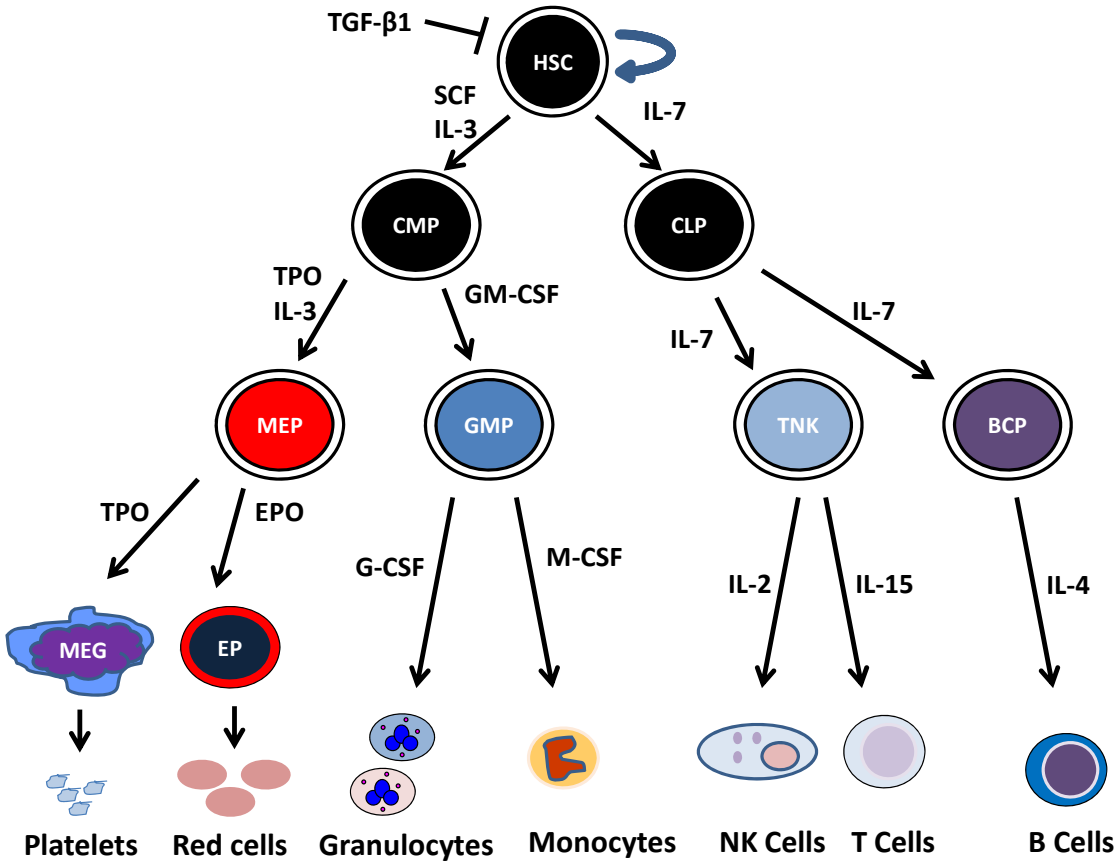


Figure 1-2 Hierarchical classification of GFs
Each GF main action in supporting the formation of blood cells of multiple and specific lineages is graphically shown in this figure. However it should be noted that because of both redundancy and pleiotropism, GFs effects on haemopoietic cells are not so specific. Abbreviations: MEP, megakaryocytic/erythroid precursor; GMP, granulocyte/macrophage precursor; TNK T cell/Natural killer (NK) precursor; BCP, B cell precursor; M-CSF, macrophage colony stimulating factor

1.1.2.1 Regulators of HSPC proliferation and myeloid differentiation: IL-3, GM-CSF and G-CSF

The main GFs involved in HSPC proliferation and myeloid development are IL-3, GM-CSF and G-CSF. IL-3 and GM-CSF have the broadest target specificity. IL-3 plays a role in the production of all myeloid cells including macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes, and erythroid cells and also of lymphoid cells as it exerts its effects mainly on the HSC and the CMP. GM-CSF acts mainly on the CMP and the granulocyte-macrophage progenitors (GMP) and thus has predominant effects on the production of granulocytes and monocytes/macrophages. Both IL-3 and GM-CSF are glycoproteins of molecular weight ranging between 14 and 30kDa depending on the level of glycosylation. Interestingly their genes are both located in tandem on the long arm of chromosome 5 and have a similar structure suggestive of a common evolutionary origin and in fact a degree of similarity in their structure is present[39]. The major source of production of IL-3 is T lymphocytes[40] although production has also been shown by natural killer (NK) cells, eosinophils and stromal cells. GM-CSF is instead equally produced by T lymphocytes, monocytes, endothelial cells, fibroblast, stromal cells of different types and also mature neutrophils[39]. Interestingly both IL-3 and GM-CSF share the same family of receptors, the gp140 family of cytokine receptors[41]. These receptors are heterodimers composed of an α chain which is the specific ligand binding chain and a β chain (also named CSF2RB) which is the signal transducing subunit and is shared amongst different GFs, namely IL-3, GM-CSF and IL-5[42]. The IL-3 receptor has been detected mainly on primitive and more committed progenitor cells with lower levels of expression on the HSC[43,44]. Similarly the GM-CSF receptor has been detected on HPC with undetectable levels on the most primitive and clonogenic stem cells[45]. This correlates with the slightly different, although still overlapping, effects of these two GFs. In fact IL-3 promotes the production from HSPC of multilineage colonies with higher replating efficiency compared to GM-CSF which mainly induces production of single lineage colonies. These results suggest that human MPP that respond to GM-CSF represent a small subpopulation of those sensitive to IL-3 and that GM-CSF effects are more prominent on more committed progenitors[46,47]. Similar effects were also demonstrated in *in vivo* experiments where administration of IL-3 stimulated haemopoiesis and induced a rise in all blood cell types and splenomegaly[48]. Significant functional redundancy between different GFs involved in the myeloid compartment proliferation and differentiation was further confirmed *in vivo* as IL-3 ligand or receptor deficient mice show a viable phenotype with some dysfunction in the eosinophils production and function, but no major effects on the HSC compartment[49].

One function peculiar to GM-CSF, compared to IL-3, is its influence on the biological functions of terminally differentiated effector cells such as neutrophils, eosinophils and macrophages. These consist mainly in production of reactive oxygen species (ROS), induction of phagocytosis and overall stimulation of an inflammatory/immune response[39].

G-CSF plays a key role in growth, differentiation, survival and activation of neutrophils and their precursors. It has also been shown to support proliferation of more primitive haemopoietic progenitors in combination with IL-3. It is a glycoprotein of about 20kDa and is produced by a variety of cells with monocytes and macrophages being the main producers. Moreover its production by fibroblasts, T lymphocytes and endothelial cells has been shown particularly following inflammatory stimuli, such the proinflammatory cytokines TNF-α, IFN-γ and IL-1[39]. The G-CSF receptor is a member of the type I cytokine receptor superfamily which includes also receptors for EPO and growth hormone. These receptors form homodimers upon ligand binding and are primarily expressed on neutrophils progenitors and mature neutrophils[50]. G-CSF plays a central role in the terminal differentiation of haemopoietic cells towards the granulocytic pathway. In fact while IL-3 and GM-CSF were capable of supporting production of both multilineage and different single lineage colonies from haemopoietic progenitors, G-CSF in the same culture was only able to support single lineage colony formation, almost exclusively of the granulocytic type[46]. Its essential role in increasing myelopoiesis and maturation of neutrophils is particularly noticeable under stress conditions (i.e. infections, cancer) when G-CSF concentration increases dramatically leading to the well recognised physiological neutrophilic response often seen during the acute phase of an infection[51]. The neutrophilia seen in these circumstances is secondary to increased production/enhanced survival of neutrophils and a shortened maturation together with increased release from the BM. In addition to this, G-CSF also has profound effects on the biological functions of neutrophils, again stimulating ROS production and their phagocytic and chemotactic activity[50]. Finally G-CSF can also influence other cell types as demonstrated by its ability to mobilise HSC. As a result of its effects on neutrophil progenitors and neutrophil development and function, G-CSF is one cytokine which has found a widespread clinical application and is currently routinely used in the management of post-chemotherapy or congenital neutropenia as well as when treating infection in patients with neutropenia or neutrophil dysfunction[39,52].

The main biological effects at the cellular level of the above mentioned GFs are therefore an increase in survival and proliferation of HSPC with a concomitant maturation of the

same towards the myeloid compartment. While the exact contribution of GFs in the lineage commitment of haemopoietic progenitors has still been questioned (see above the stochastic model for cytokine function), and the exact signals causing lineage fate decisions are unclear, a better understanding of the survival and proliferative signals elicited by these GFs is available. Because both the common β chain receptor of IL-3 and GM-CSF, which is the signal transduction subunit, or the G-CSF receptor do not have tyrosine kinase (TK) activity, their ability to transduce signals following GF stimulation is secondary to the presence of intracellular TK which are activated upon ligand binding and in turn phosphorylate tyrosine residues within the GF receptor and other signalling molecules[39]. Amongst those, the janus kinase (JAK) family of proteins plays a prominent role in all three aforementioned GF signalling mainly via activation of the signal transducer and activator of transcription (STAT) transcription factors. Moreover two other survival signalling pathways, namely the PI3 kinase/AKT and Rat sarcoma (RAS)/Mitogen activated protein (MAP) kinase pathways are activated by the same GF receptors via the phosphotyrosines produced on the receptors themselves or intracellular adaptor proteins upon ligand binding[41]. In the next sections a more detailed analysis of these GF receptors activated signalling pathways will be presented.

1.1.2.1.1 The JAK/STAT pathway

The JAK/STAT pathway is central to haemopoiesis and the most important pathway in the normal cellular response to a variety of GFs. JAKs are intracellular TKs which, following dimerisation of GFs receptor upon binding to their ligands, are brought close together and become activated through auto- and cross-phosphorylation. Active JAKs are then able to phosphorylate the GFs receptor creating docking sites for the STAT transcription factors, which as a result, also get phosphorylated by the JAK proteins. Phosphorylated STATs are activated and translocate to the nucleus where they induce changes in gene expression within the target cells which are central for the growth, survival and differentiation[53] of HSPC into different lineages, such as the proliferative genes *cyclin D1*[54], *cyclin D2*[55] and the antiapoptotic gene *BCL-X_L*[56] (figure 1-3). The JAK/STAT pathway is tightly regulated normally by negative feedback mechanisms mainly via the activation of the cytokine-induced SH2 containing (CIS) and the suppressors of cytokine signalling (SOCS) family of proteins. These proteins exert their negative effects by direct kinase inhibition and/or competitive binding to phosphotyrosine docking sites on the GFs receptors which prevents binding on the same site by stimulatory pathways signalling proteins[57].

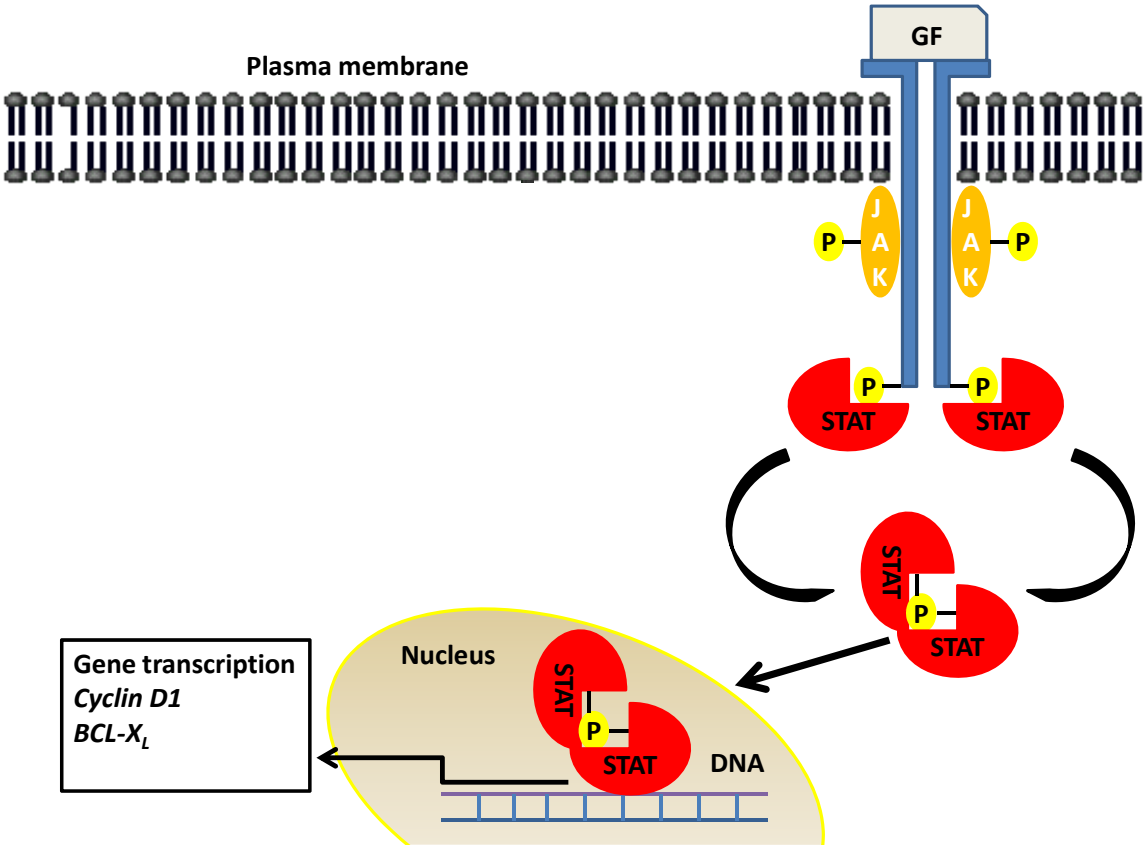


Figure 1-3 The JAK/STAT pathway

There are 4 JAK proteins in mammals: JAK1, JAK2, JAK3 and TYK2. Each of them is activated by various different GFs and can in turn activate different types of STATs and therefore plays a rather broad role in normal haemopoiesis. Specific *Jak* gene knockout (KO) experiments in mouse models have helped clarifying this and narrowed down the most prominent role in haemopoiesis for each JAK (table1-2).

KO mouse	***Phenotype***	***GFs affected***
Jak1	Impaired lymphopoiesis	IL-2, IL-4, IL-6
Jak2	Impaired erythropoiesis/myelopoiesis	EPO, IL-3, GM-CSF
Jak3	Defective lymphopoiesis/dysregulated myelopoiesis	IL-4, IL-7
Tyk2	Defective adaptive and innate immune responses	IFN α/β/γ, IL-12

Table 1-2 Role of *Jaks* in haemopoiesis based on phenotypes of KO mouse (Adapted from[57])

Seven STAT transcription factors have been described in mammals: STAT 1 to 6 and STAT5a and STAT5b which show extensive sequence homology. They are activated via JAKs by different GFs receptors in a more specific manner, although different receptors can still activate a common STAT. Again *Stat* KO mouse models have helped clarify the main role for each of them in normal haemopoiesis (table1-3).

KO mouse	*Phenotype*	*GFs affected*
Stat1	Defective IFN responses	IFN α/β/γ
Stat2	Defective type I IFN responses	IFN α/β
Stat3	Embryonic lethal/impaired T cell proliferation	IL-2, IL-6, IL-10
Stat4	Impaired Th1 cell development	IL-12
Stat5ab	Reduced granulocytic proliferation	IL-3, GM-CSF, G-CSF
Stat6	Impaired Th2 differentiation	IL-4

Table 1-3 Role of *Stats* in haemopoiesis based on phenotypes of KO mouse (Adapted from[57])

Both *in vivo* (see table 1-2 and 1-3) and *in vitro*[58,59] work has shown that both IL-3 and GM-CSF signal mainly (although not exclusively) via JAK2/STAT5. The JAK2/STAT5 axis also plays a central role in G-CSF induced signalling, although other members of both families (such as JAK1 and STAT3) have also been involved in G-CSF signalling[60]. Interestingly JAK2 also has a prominent role in EPO signalling and therefore erythroid development[61].

JAK2 therefore plays a central role in the normal cellular response to haemopoietic GFs particularly of the myeloid and erythroid lineages and in fact *Jak2* KO mice are embryonic lethal because of absence of definitive erythropoiesis. *Jak2* KO foetal liver cells are unable to respond to EPO, IL-3 and GM-CSF and show a clear defect in erythroid/myeloid lineage differentiation with little effect on lymphocytic lineage differentiation thus confirming *Jak2*'s essential role for erythroid and myeloid cell development due to its central role in erythropoietic and myelopoietic GFs receptor signalling. Interestingly however no effect was seen on the development of LT-HSC in the same mice which might be secondary to a degree of redundancy with other cytokines involved in HSC maintenance[62,63].

JAK2 transduces GFs signals through activation of its downstream target STAT5 and in support of a role for STAT5 in haemopoiesis, a constitutively active *Stat5* mutant was able to compensate for loss of *Jak2* in mouse haemopoiesis *in vivo*[64]. However the first *Stat5* KO mice described showed no effect on red cell production and a mild reduction in granulocytic differentiation[65,66]. These mice however expressed a hypomorphic N-terminally truncated *Stat5* allele. When complete ablation of *Stat5* was achieved a more severe phenotype was elicited with perinatal lethality, although up to 1% of animals survived till 6 weeks[67,68]. Moreover in transplantation assays, *Stat5* null HSC showed only modest defects in the myeloerythroid repopulation[69]. Taken together these results suggest that *Stat5* deletion phenotype on myeloerythroid development is overall less severe than the one shown by *Jak2* KO mice. This would support the hypothesis that additional signalling pathways are activated by JAK2 beside STAT5 activation to support myeloid

and erythroid cell development. Conversely, recently it has been shown that STATs activation might also be dependent on other kinases, such as those of the family homologous to the Rous sarcoma virus gene (SRC), thus adding further complexity to this system and the relative contribution of STAT and JAK to GFs response[70].

1.1.2.1.2 The PI3 kinase/AKT pathway

The PI3 kinase/AKT pathway is another pathway activated by several GFs including IL-3[71,72], GM-CSF[73] or G-CSF[74]. PI3 kinase consists of a family of related proteins with similar catalytic function. Although 3 classes of PI3 kinases have been identified (class I, II, III), class I PI3 kinase (from now on referred to as PI3 kinase for simplicity) is the most studied and relevant to the haemopoietic cells[75]. PI3 kinase is a heterodimeric protein consisting of an 85kDa regulatory subunit containing the phosphotyrosine binding domain SH2 and SH3, and 110kDa catalytic subunit[76]. The regulatory subunit allows the heterodimer to bind phosphotyrosines which appear on surface receptors or receptor associated proteins such as p80[77] following haemopoietic GFs stimulation[78]. As a result PI3 kinase translocates near the plasma membrane, is activated and through its catalytic subunit promotes the conversion of the membrane lipid component phosphatidylinositol - 4,5- bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 then acts as a docking site for protein kinases containing the so called pleckstrin homology domains, such as the phosphoinositide dependent kinase (PDK) and AKT[79]. AKT can be activated by direct binding to PIP_3[80] and through phosphorylation by PDK[81]. Following some GFs receptor activation, such as G-CSF, AKT appears to be activated by SRC kinases[74]. Different isoforms of AKT (AKT 1, 2 and 3) have been reported and its most relevant biological activities in the haemopoietic system are, as for the other pathways, in regulating cell cycle progression and apoptosis[75]. In particular its antiapoptotic effects are normally accomplished through the phosphorylation and consequent inactivation of different targets, including the proapoptotic proteins such as BAD[72,82] and caspase-9[83]. Amongst the AKT targets, the transcription factors of the forkhead box subgroup O (FOXO) family are particularly important given their pivotal role in normal regulation of cell proliferation, metabolism and survival[84]. These transcription factors (in particular FOXO3a in the haemopoietic cells) normally promote expression of genes involved in cell cycle arrest and/or apoptosis, such as the CKIs *Cdkn1a/p21*[85], *Cdkn1b/p27*[86] and the proapoptotic protein BIM[71], while repressing the expression of proteins involved in cell cycle progression such as the D cyclins[87,88]. Following their phosphorylation by AKT, FOXOs are relocated in the cytoplasm and become inactive[89] which results in inhibition of

the FOXO apoptotic and cell cycle arrest induced programme. The PI3 kinase/AKT pathway is normally regulated via the action of specific phosphatases, such as phosphatase and tensin homolog (PTEN) phosphatase, that removes the 3-phosphate from PIP_3, thus preventing AKT activation[90]. More recently two other phosphatases, SHIP 1 and SHIP2, have been also shown to act as negative regulators of the pathway through removal of the 5-phosphate from PIP_3[75](figure 1-4).

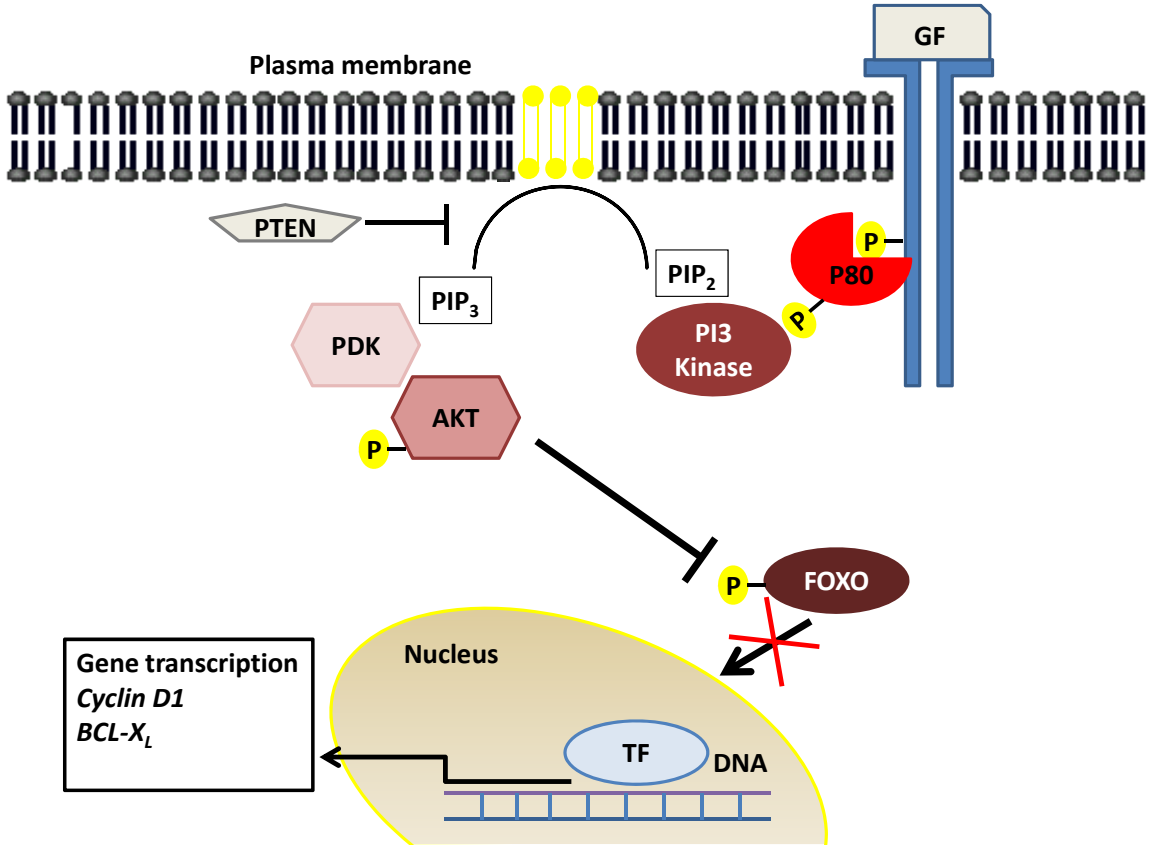


Figure 1-4 The PI3 kinase/AKT pathway
Activation of PI3 kinase and its downstream effector AKT has multiple effects on signalling. Effects on FOXO transcription factors are specifically shown which result in inhibition of FOXO activity and therefore absence of their negative regulatory effects on other transcription factors (TF) regulating proliferative and prosurvival genes.

1.1.2.1.3 The RAS/MAP kinase pathway

The RAS/MAP kinase pathway is another central signal transduction pathway which relays signals from cell surface GFs receptors (including IL-3 and GM-CSF) to transcription factors in the nucleus[91]. RAS is a small guanosine triphosphate (GTP) binding protein that is active when bound to GTP and inactive when bound to guanosine diphosphate (GDP). Switching between these states is determined by changes in its conformation following GFs stimulation. These conformational changes allow alternatively the binding of GTPase activating proteins and the guanine exchange factors, which respectively remove a

phosphate from GTP and inactivate RAS, or activate it by addition of a GTP molecule[75]. RAS activation by GFs is normally secondary to the binding on GFs receptors of adaptor molecules such as SHC[92] and GRB2, which in turn activates the guanine exchange factor SOS leading to RAS activation[93]. Active RAS recruits the serine/threonine kinase RAF to the cell membrane, which in turn activates the signalling cascades through intermediate kinases called MAP kinases, such as mitogen extracellular kinase (MEK). MEK phosphorylates and activates in turn the extracellular signal regulated kinase (ERK) which then phosphorylates and activates transcription factors, such JUN, FOS and MYC leading to the transcriptional regulation of several genes involved in proliferation and apoptosis[75] (figure 1-5).

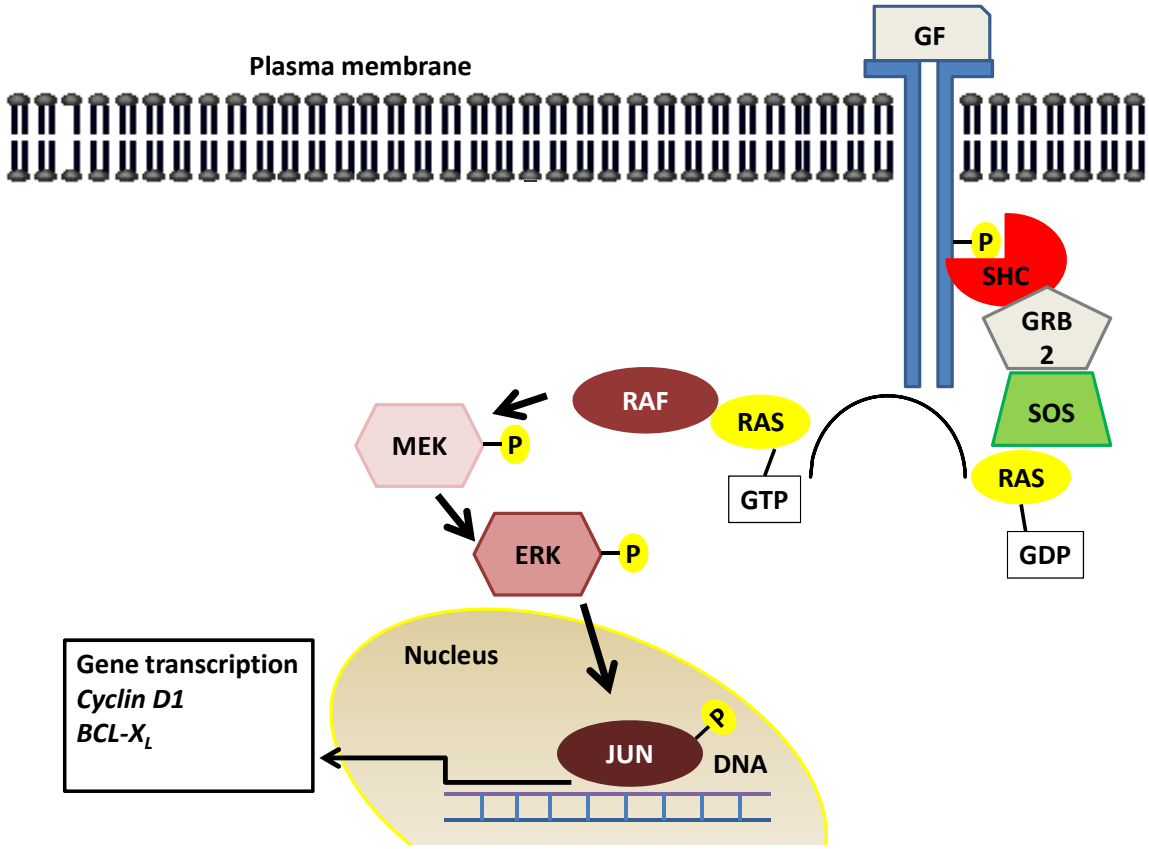


Figure 1-5 The RAS/MAP kinase pathway

1.1.2.2 Regulators of HSC quiescence and maintenance: TGF-β1 and neurotransmitters

HSC are predominantly quiescent. Quiescence, as discussed earlier, is essential to preserve HSC self-renewal potential (maintenance). Recently using a mouse model it was shown that amongst the most phenotypically primitive HSCs, the quiescent population was the only one able to reconstitute haemopoiesis in serial transplants[94]. This population of quiescent cells acts like a reservoir which normally does not contribute to daily blood production, but is only called upon under stress situations and as such it represents a safety net to prevent stem cell exhaustion during the life of an adult organism. Therefore quiescence and maintenance are two attributes of HSC closely linked as also shown by the HSC exhaustion observed in mice carrying deletion of genes central to HSC quiescence, such as the cell cycle negative regulators CKIs *Cdkn1a/p21*[95] and *Cdkn1c/p57*[96]. Quiescent HSCs are located in specific areas within the BM, particularly along the endosteal lining in the cavities of trabecular bones. These areas of the BM (called the BM niche) are able to provide the best environmental cues for HSC quiescence[31,32]. These cues can be soluble proteins acting in a paracrine way or membrane bound proteins acting instead by direct interaction between niche cells and HSC. Several of these cues have been described over the years and the list is rapidly expanding. Amongst the extrinsic regulators of normal HSC quiescence/maintenance it is worth mentioning TGF-β1[27,97], TPO[98,99], Wnt/β-catenin[100-102], Hh[103,104], Notch[31], integrin[105], chemokine signalling[27,106,107] and more recently also certain types of neurotransmitters[108]. Given the fact that they were part of the investigations presented in this thesis, a more in depth analysis of the role of TGF-β1 and neurotransmitters in HSC quiescence and maintenance will be presented in the following sections.

1.1.2.2.1 TGF-β1 role in HSC quiescence and maintenance and its mechanisms of action

TGF-β1 is a cytokine produced by almost every known cell type, including HSC, and has a role in the regulation of most tissues' development and renewal. In haemopoiesis its main role is as a negative regulator of HSC proliferation, but TGF-β1 has been noted to have pleiotropic effects which can vary depending on the cell context[109]. The TGF-β1 peptide is a 25kDa peptide which is normally released as a latent complex which includes the TGF-β1 peptide bound to a latency associated protein (small latent complex). This small latent complex is then bound to a latent TGF-β1 binding protein (large latent complex) which allows this complex to associate with the extracellular matrix. TGF-β1 in a large latent

complex (also called latent TGF-β1) is inactive and therefore its function is modulated by its activation which requires the dissociation of the TGF-β1 peptide from the latency associated protein normally via a proteolytic process[110]. It has been shown that HSCs are unable to activate TGF-β1 by themselves and require BM niche cells to activate it[97,111].

Two receptors for TGF-β1 have been described: the TGF-β1 receptor type 1 (TGF-β1 R1) and type 2 (TGF-β1 R2). These are serine/threonine kinases that, upon ligand binding, heterodimerise and activate their kinase activity leading to their phosphorylation and downstream signalling activation.

TGF-β1 biological function in haemopoiesis is to induce quiescence in HSC thus preserving their cloning and repopulating potential. This has been shown in several *in vitro* and *in vivo* experiments. TGF-β1 has been shown to reduce the proliferation of LTC-IC and of enriched $CD34^+$ $CD38^-$ human haemopoietic cells. Interestingly its effects were different on the more mature progenitors ($CD34^+$ $CD38^+$) with even stimulation reported, thus confirming its pleiotropic effects which are highly dependent on the cell context[112-114]. Interestingly the effects on quiescence correlated with a higher repopulation capacity as human HSCs treated with TGF-β1 *in vivo* showed markedly increased repopulating capacity in secondary recipients[27]. Similarly *in vitro* treatment with TGF-β1 induced quiescence but also preserved the ability to produce multilineage colonies and repopulating capacity in competitive transplantation assays when compared to untreated (UT) cells. Moreover this was not associated with loss of viability or apoptosis induction in the HSC[97,115]. However it has also to be noted that mice deficient in *Tgf-β1 R1* show no defect in HSC maintenance and quiescence[116]. It is therefore possible that a degree of redundancy exists, both in some components of the TGF-β1 signalling and overall in quiescence regulation which prevents the development of a clear phenotype *in vivo*.

TGF-β1 can activate several signalling pathways depending on the cell context. However the two main pathways activated in the induction of quiescence and stem cell maintenance are the SMAD and AKT/FOXO pathways. The SMAD signalling pathway is the canonical pathway activated by TGF-β1. In simple terms, the activation of the TGF-β1 receptors upon ligand binding leads to the phosphorylation of the receptor regulated SMADs (SMAD 2 and SMAD3). Once phosphorylated, these can interact with SMAD4 to form an active transcriptional complex able to regulate the expression of several genes. The SMAD transcriptional complex is not in itself particularly strong as often the SMAD binding motif on the target genes are scarce. This practically means that for TGF-β1 to elicit a response

other cofactors have to be present and therefore the nature of response to TGF-β1 is highly dependent on the type of cofactors present in each cell. This is currently considered a possible explanation for the highly variable response to TGF-β1 observed in different cell contexts[117]. In terms of the cytostatic response to TGF-β1, this appears mainly secondary to the ability of TGF-β1 to activate CKIs, such as CDKN1A/p21, CDKN1B/p27 or CDKN1C/p57, while also being able to repress growth promoting factors, such as MYC[118]. Interestingly FOXO transcription factors have been shown to act as SMAD cofactors in TGF-β1 induction of CDKN1A/p21[85] (figure 1-6).

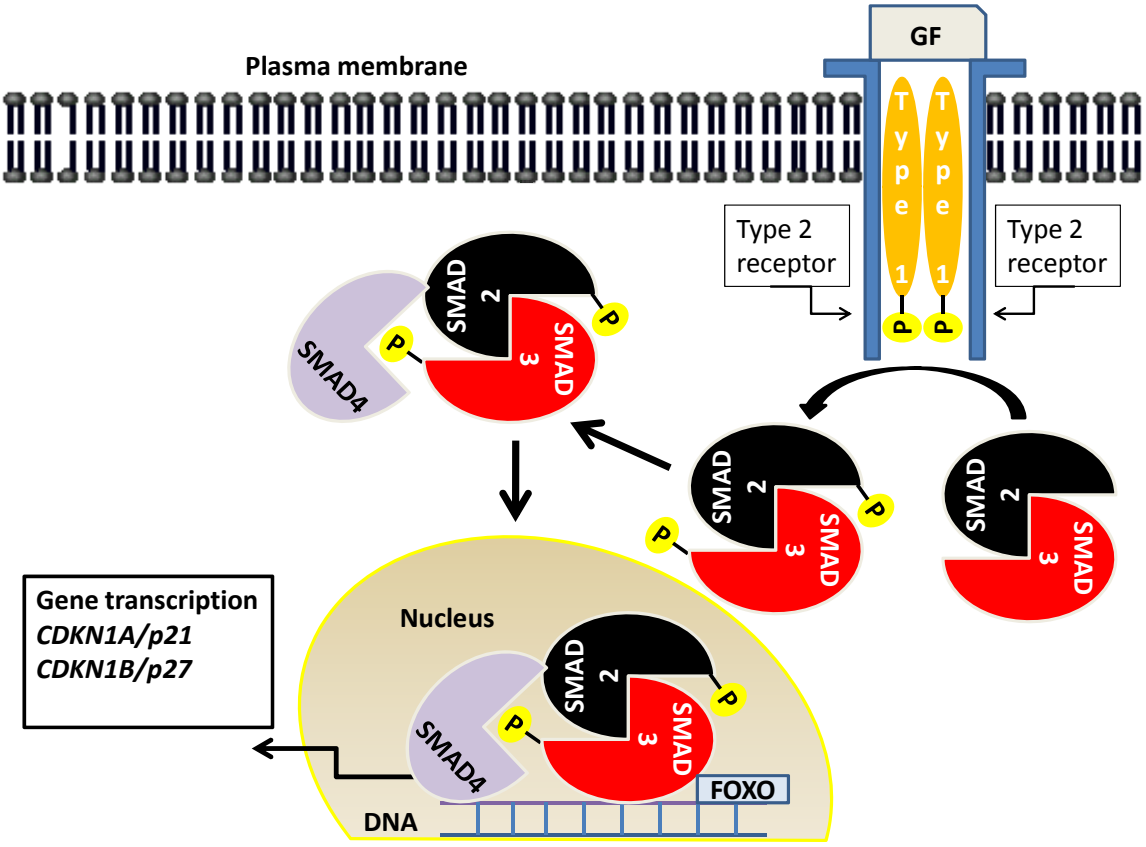


Figure 1-6 The TGF-β1/SMAD pathway

FOXO role in inducing cell cycle arrest and also apoptosis has already been discussed in the context of the PI3 kinase/AKT signalling. It is worth noting here that TGF-β1 has been shown to induce FOXO signalling by inhibiting AKT activity. The exact mechanism of AKT inhibition by TGF-β1 is not clear but recently a possible mechanism in the haemopoietic cells has been described involving its ability to prevent lipid raft clustering on the cell membrane of HSC[97]. Lipid rafts act as platforms for cellular functions such as cytokine signalling. Cytokine stimulation can induce lipid raft clustering which concentrates cytokine receptors and downstream signal transducers within the cluster and

further activates cytokine signalling thus controlling signalling and functional outcomes. In HSC lipid rafts have in fact been shown to increase AKT signalling leading to FOXO nuclear exclusion and inactivation[119]. Therefore TGF-β1 might be able to target both the SMAD and AKT/FOXO pathways and allow their interaction which appears to be necessary to regulate the expression of genes involved in stem cell quiescence, such as *CDKN1A/p21*. In this respect it is also worth noting that both *Foxo*[120] and *Smad4*[121] have been shown to play a key role in HSC maintenance in KO mouse models, which at least in the case of FOXO was secondary to reduced quiescence and increased apoptosis possibly secondary to excessive accumulation of ROS[122].

1.1.2.2.2 The putative role of neurotransmitters in HSC maintenance

The term neurotransmitters or neuromediators normally refers to a broad group of endogenous chemicals that transmit signals from a neuron to a target cell across a synapse. Neurotransmitters are normally stored in synaptic vesicles clustered in the nerve axon terminal and are released into the synaptic cleft following electrical nerve stimulation by depolarisation. Classical neurotransmitters, such as norepinephrine (NEPI), serotonin (5HT) and acethylcoline (ACH) are usually synthesised from simple molecule precursors, such as amino acids, through few biosynthetic steps. A common classification of neuromediators is based on their chemical structure which allows their differentiation into 3 main groups, including the biogenic amines, such as ACH, NEPI, dopamine, 5HT, histamine (HIS), the amino acids, such as glycine, glutamate (GLU), aspartate, and the peptides, such as somatostatin, opioid peptides and others[123,124]. Although neurotransmitter function has been mainly studied in relation to the nervous system, back in 2001 it was showed that an overlapping gene expression profile could be detected between mouse neuropoietic and HSC which included seven transmembrane domain receptors commonly used by neuromediators in relaying their signals[125]. More recently a report specifically looked at gene and protein expression for the same receptors in human HSPC and found that indeed several neurotransmitter receptors were expressed on HSPC, including opioid and 5HT receptors. These receptors were functionally active and interestingly their expression levels were higher in the more primitive population ($CD34^+$ $CD38^-$). This provided the first evidence for a common signalling mechanism between nervous and haemopoietic systems[126]. As already mentioned, HSC, particularly the most primitive ones, reside in specialised BM niches where they are subject to numerous cues through contact with stromal cells via adhesion molecules and soluble proteins. Nerve fibers have also been reported as being part of the BM niche. The role of the nervous system in regulation of

HSC maintenance, proliferation and migration has however only recently started to be unveiled. In murine models, the sympathetic nervous system, through the neurotransmitter NEPI, has been shown to regulate HSC egress from the BM by suppressing endosteal osteoblast function and leading to reduction in the levels of a key chemokine involved in HSC BM retention, SDF1[127]. Moreover treatment of human $CD34^+$ cells with catecholaminergic neurotransmitters, such as dopamine and NEPI, has been shown to increase their mobilisation, proliferation and repopulation capacity of immunodeficient hosts[108,128], while 5HT has been shown to enhance *ex vivo* expansion of cord blood $CD34^+$ cells, including cells capable of repopulating immunodeficient mice BM[129]. A very recent report has also shown an indirect role of specific peripheral nervous system cells, the non-myelinating Schwann cells, in regulating haemopoietic cells maintenance through regulation of the activation process of latent TGF-β1 into its active form[130]. Taken together these results support a model where the autonomic nervous system regulates HSC function in several different ways, through both direct and indirect effects. According to this model a dynamic crosstalk exists between the BM niche stromal cells, the HSC and the nervous system, which create a regulatory "brain-bone-blood triad", as it has been named, whose primary role is to regulate HSC maintenance, proliferation and BM homing[131].

Little is known as to the mechanisms used by the aforementioned neurotransmitters to determine their effects on the HSC. Most neurotransmitters relay their signals through several different pathways using different receptors. Amongst those the already mentioned seven transmembrane domain receptors (also called serpentine receptors) play the main role. These receptors constitute the so called G protein coupled receptor family. Upon ligand binding to these receptors, the coupled G proteins become active as they bind a molecule of GTP. G proteins are heterotrimeric proteins consisting of an α, β and γ subunit. They are classified on the basis of their α subunit which is mainly responsible for the stimulation or inhibition of their target effector proteins which are normally an enzyme like adenylyl cyclase and phospholipase C β1 (PLCB1) or an ion channel. There are several subtypes of Gα proteins, with the main ones being G_s, G_i and G_q. $G_{s,}$ stimulates, while G_i inhibits, adenylyl cyclase which is the enzyme that regulates intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP), by converting adenosine triphosphate (ATP) into cAMP. G_q stimulates PLCB1 which is the enzyme that hydrolyses the phospholipid component of the plasma membrane, PIP_2 into inositol 1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG). IP_3 promotes the release of sequestered calcium (Ca^{2+}) from intracellular stores into the cytoplasm where it acts as a second messenger, while DAG activates protein kinase C (PRKC). Ion channels can also be

activated by both G_i and G_q and increase intracytoplasmic levels of several ions including Ca^{2+} and potassium[123,132]. Interestingly in the report showing neurotransmitter receptors expression on HSC, their functionality was also demonstrated by measuring intracellular cAMP levels following agonist binding[126] (figure 1-7).

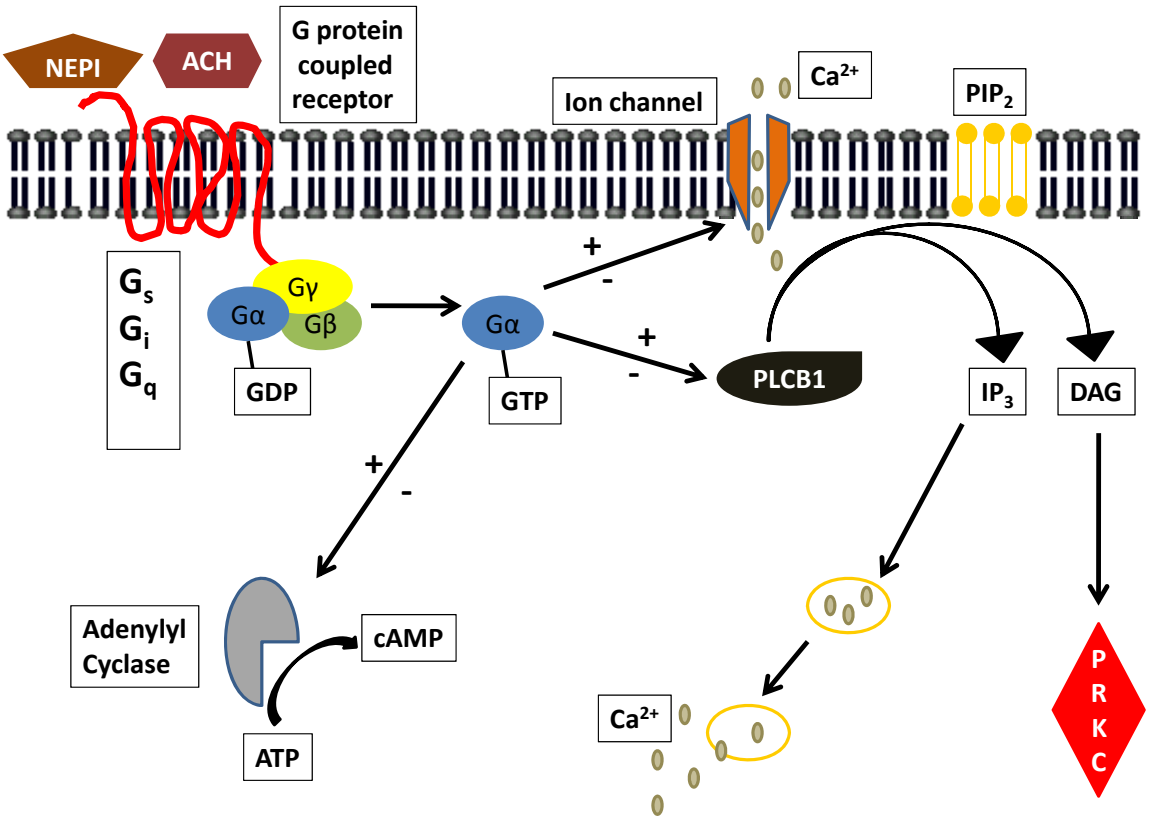


Figure 1-7 The G protein coupled receptor signalling pathway
Several neuromediators such as NEPI and ACH activates G protein coupled receptors. These in turn can stimulate or inhibit several different signalling pathways depending on the type of G protein they are coupled too. '+' indicates stimulation and '–' inhibition. See text for full explanation of the pathways and abbreviations.

How these signals determine the biological outcome seen following neuromediators stimulation is still unclear. A possible role for the canonical Wnt pathway through stabilisation of β-catenin has been suggested following NEPI stimulation[108]. The Wnt/β-catenin pathway has a well defined role in HSC self-renewal and maintenance. Treatment of single mouse HSC with Wnt ligand caused an increase in their self-renewal as demonstrated both phenotypically and functionally in transplantation assays and this was secondary to β-catenin activation[101]. Similarly upregulation of β-catenin activity through transduction of a constitutively active β-catenin in mouse HSC led to their increased self-renewal and repopulating capacity of irradiated hosts, while the opposite results were seen upon inhibition of β-catenin activity through transduction of its inhibitor axin in HSC[102].

In conclusion the study of the role of the nervous system in regulation of HSC function has only recently found some momentum. While evidence is accumulating on the role of several neurotransmitters in HSC self-renewal, mobilisation, maintenance and quiescence and its possible mechanisms, the exact role of each neurotransmitter, their interaction with other regulators and other possible mechanisms underlying their effects are an area of current investigation.

1.1.2.3 Pleiotropic regulators of haemopoiesis: TNF-α

TNF-α is a cytokine produced primarily by blood cells of the immune system, such as macrophages, NK cells and lymphocytes, although production by other cell types, in both physiological and pathological conditions, has been reported. It is a trimeric protein of approximately 17kDa which can be expressed in both transmembrane and soluble forms. It can bind to two different receptors, TNF-α receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2). TNFR1 is expressed on every cell type known to date and is essential for the transduction of most of the signalling pathways activated by TNF-α. It contains a death domain which, despite its name, is able to relay not only proapoptotic signals (such as caspase-3 and 8 activation), but also prosurvival signals, mainly through the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and MAP kinase pathway. Conversely TNFR2 expression is limited to immune cells, endothelial cells and nerve cells and although reports have suggested a role mainly in transducing proliferative signals through NFκB and MAP kinase, it seems to have overlapping function with TNFR1 with which it acts cooperatively[133,134].

The role played by TNF-α in normal haemopoiesis has been extensively investigated with conflicting results. Although usually considered a negative regulator of haemopoiesis, TNF-α has been shown *in vitro* to both inhibit and promote growth of human HSPC partially depending on the maturation stage of the target cells, its concentration and other GFs involved[135,136]. To summarise the current evidence, it appears that TNF-α is able to stimulate the proliferation and expansion of the more primitive human haemopoietic cells ($CD34^+$ $CD38^-$) particularly when the latter are cultured in the presence of high concentrations of GFs, such as IL-3 and GM-CSF, and with maximal effects seen when TNF-α is used in a concentration range of 0.1 to 5ng/mL, while higher concentrations show inhibitory effects[135,137-139]. These proliferative effects are also associated with a more rapid differentiation of HSC and a subsequent downstream differentiation induced growth arrest. As a result the effects of TNF-α on more mature and committed progenitors are

rather inhibitory than stimulatory[140,141]. Similarly *in vivo* experiments using single and double KO mouse for both TNF-α receptors have shown increased BM cellularity, possibly explained by a tendency towards increased sizes of both mature and immature progenitor cell populations. This would suggest release of inhibitory effects from TNF-α on mature progenitors. The end result of these effects on the overall maintenance of LT-HSC is however not clear and some reports have suggested that by pushing HSC into differentiation TNF-α would determine a negative effect on LT-HSC maintenance[142]. Overall conflicting results on TNF-α effects on LT-HSC maintenance have been reported which could not be easily reconciled[143,144]. TNF-α can also exert its effects on haemopoiesis indirectly, i.e. by stimulating HSPC and other cells from the microenvironment to produce GFs involved in HSPC proliferation and differentiation (such as GM-CSF) [145,146] or by upregulating their cognate receptor expression[147] (i.e. CSF2RB). In conclusion it would appear therefore that the effects of TNF-α on normal haemopoiesis are more complex than usually thought and testify as to the pleiotropic and cell context dependent effects of this GF.

TNF-α is able to activate multiple signalling pathways with different biological outcomes which helps to explain its pleoiotropic effects. Moreover it often activates them simultaneously so that their net balance determines its biological effects. Amongst the signals activated, the apoptosis/caspase, the MAP kinase and the NFκB pathways are particularly relevant. The apoptosis signal is relatively simple as upon TNF-α binding, TNFR1 recruits a protein called TRADD through its death domain which can in turn recruit another protein called FADD leading to sequential activation of caspase-8 and 3 and apoptosis[148]. It is a relatively rapid signal not requiring protein synthesis. The MAP kinase pathway is also activated by TNFR1 and TRADD, but through a different intermediate called TRAF2[134]. Therefore RAS/RAF proteins are not required for MAP kinase activation by TNF-α. However once the MAP kinases are activated via TNFR1/TRADD/TRAF2, the signalling cascade is similar to the one previously described in relation to other GFs signalling. A more detailed description is instead required for the NFκB pathway and its activation by TNF-α.

1.1.2.3.1 The NFκB pathway

NFκB comprises a family of five transcription factors (p65 REL-A, REL-B, C-REL, p50 NFκB and p52 NFκB) which despite their name are present in virtually every cell type. These proteins can form a variety of homo or heterodimers, however, due to the presence

of a strong transcriptional activation domain, p65 REL-A is responsible for most of the transcriptional activity of NFκB. The classic heterodimer comprising p65 REL-A and p50 NFκB is the most abundant and ubiquitous in mammals and the one transducing most of the proproliferative, antiapoptotic and inflammatory signals of NFκB family following its activation[149,150]. Because of this, the following discussion will be mainly focused on the p65 REL-A/p50 NFκB dimer which from now on will be referred to as NFκB for simplicity.

The regulation of NFκB is based on its intracellular localisation. In its inactive state, NFκB is bound to the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) proteins, such as IκBα, which prevent its nuclear translocation and activation. NFκB can be activated by a variety of stimuli, such as the proinflammatory GFs TNF-α and IL-1 or lipopolysaccharide produced in microbial and viral infection. Specifically in the case of TNF-α, upon ligand binding a TNFR1/TRADD/TRAF2 complex is formed, which through other intermediates (RIP), activates the IκBs kinase (IKK) complex. When active, IKK complex phosphorylates IκBs leading to their proteasomal degradation[151] and release of NFκB. Furthermore released NFκB also becomes accessible to direct phosphorylation by the IKK complex and possibly also other kinases on two key serine residues (529 and 536) of the transactivation domain of p65 REL-A[152,153]. This direct phosphorylation also plays a prominent role in the full activation of NFκB[154]. Phosphorylated NFκB is then free to shuttle into the nucleus where it can activate the transcription of several genes involved in proliferation, survival and inflammatory responses[150,155]. With regards to its effects, NFκB is well known for its role in regulating the proinflammatory responses to TNF-α by inducing the production of several inflammatory mediators, such as cytokines (IL-6, IL-8), chemokines and enzymes, such as cyclo- and lipo-oxygenase. NFκB also has strong proliferative effects, through both the direct induction of the transcription of genes involved in cell cycle progression, such as cyclin D1[156] or indirectly by inducing the production of GFs, such as GM-CSF[150]. Finally its antiapoptotic effects are mainly secondary to the induction of the so called inhibitor of apoptosis (IAP) proteins. These include 4 members named IAP1, IAP2, XIAP and survivin. They act by blocking several caspases' activation either directly (caspase-3, caspase-7) or indirectly (caspase-8 and caspase-9)[157-160] and also, particularly IAP2, by enhancing NFκB signalling through a positive feedback[161]. Other antiapoptotic proteins of the BCL-2 family, such as BCL-X_L have also been shown to be activated by NFκB[162] (figure 1-8). It is worth noting that the activation of NFκB signal by TNF-α is able to prevent its simultaneous induction of apoptosis and this has been shown also for other apoptotic stimuli able to activate NFκB[163].

Therefore despite its name, TNF-α is indeed a poor inducer of apoptosis and this is due to its ability to activate NFκB. Only when NFκB signalling is abolished/inhibited can TNF-α effectively induce apoptosis as shown by experiments in p65 *Rel-A* null cells[164].

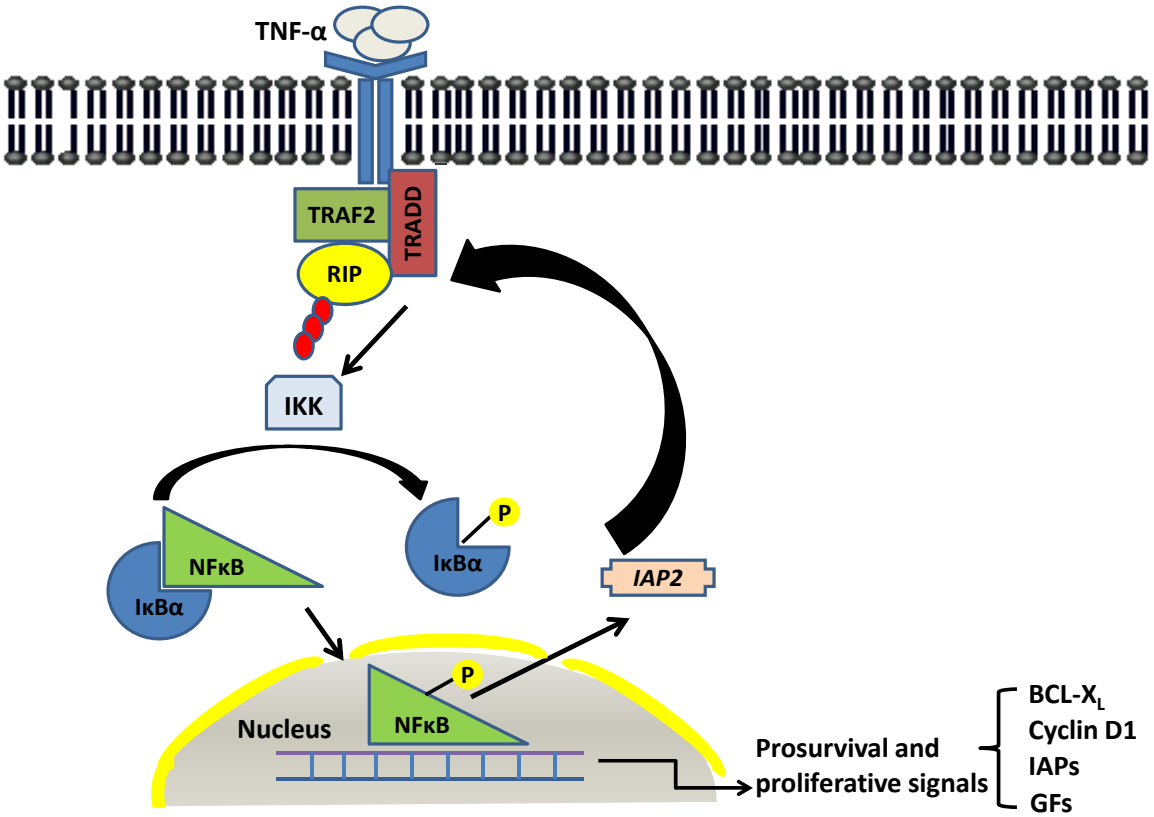


Figure 1-8 The NFκB pathway

1.2 Leukaemogenesis

1.2.1 The leukaemia stem cell (LSC)

The hierarchical organisation of the haemopoietic system coupled with the observation that many leukaemias demonstrate significant heterogeneity with respect to morphology, cell growth kinetics and response to therapeutic agents, has led to the testing of the hypothesis of whether leukaemia is also hierarchically organised. In 1971 it was shown using ascites derived mouse myeloma cells, that only 1 in 10,000 to 1 in 100 leukaemic cells were able to form colonies in standard colony-forming assays[165]. However it was not until 1997 that conclusive evidence of the existence of LSC was produced. Using transplantation assays of peripheral blood leukocytes from patients with acute myeloid leukaemia (AML) in immunodeficient mice, Bonnet and Dick demonstrated that in almost every type of AML only a population of cells expressing specific stem cell markers ($CD34^+$ $CD38^-$) was able to reproduce a disease in the host with the same features present in the donor. Moreover this same population showed self-renewal potential because of its ability to re-establish leukaemia in secondary recipients[166]. The leukaemic $CD34^+$ $CD38^-$ population therefore truly contained a LSC as it displayed both of the key properties of a normal HSC, i.e. self-renewal and proliferation/differentiation. The conclusion from this landmark study was that AML followed a hierarchical organisation similar to the one present in normal haemopoiesis and could be viewed as an aberrant haemopoietic tissue initiated by a minority of cells within the bulk of the tumour which displayed indefinite proliferation potential as a result of acquired mutations. The main source of the heterogeneity seen in AML was therefore not secondary to continuing and accumulating genetic changes within the original leukaemic clone, but rather a consequence of the aberrant and variable differentiation programme of the LSC which depends on the nature of the original transforming genetic event. As a result, a LSC gives rise to a phenotypically diverse progeny of cells with limited or no proliferative potential, forming the disease bulk, while also maintaining a small proportion of LSCs with indefinite proliferation potential and displaying little phenotypic variability. In this report the LSC was only isolated within a population sharing the same markers of normal HSC. It was therefore originally thought that normal HSCs provide a more permissive environment for LSC development because they already possess an active self-renewal machinery. Moreover the HSCs are the only cells within the haemopoietic system that persist throughout life and undergo several rounds of divisions which means that they can more easily accumulate the necessary mutations to become malignant[2,167]. In support of this model, studies have shown that

potent oncogenes such as breakpoint cluster region-abelson (*BCR-ABL*) are unable to confer self-renewal properties to murine progenitors[168]. However over the last decade evidence has accumulated that this is not always the case and a LSC does not always need to originate from a normal HSC but might also arise in a more mature cell that has reacquired gene expression and functional characteristics that support self-renewal. In fact in contrast with *BCR-ABL*, other oncogenes such as *MLL-AF9, MLL-ENL* and *MOZ-TIF2* were all capable of conferring properties of LSC to murine committed HPCs which normally lack self-renewal capacity[168-170]. More recently it has also been shown in some forms of human AML that phenotypic progenitors ($CD34^+$ $CD38^+$) or even $CD34^-$ cells acquire self-renewal potential and can act as LSC[171,172]. Therefore there appears to be a higher variability in the phenotype of LSC than previously realised. A model for origin of LSC, including both scenarios, is shown in figure 1-9.

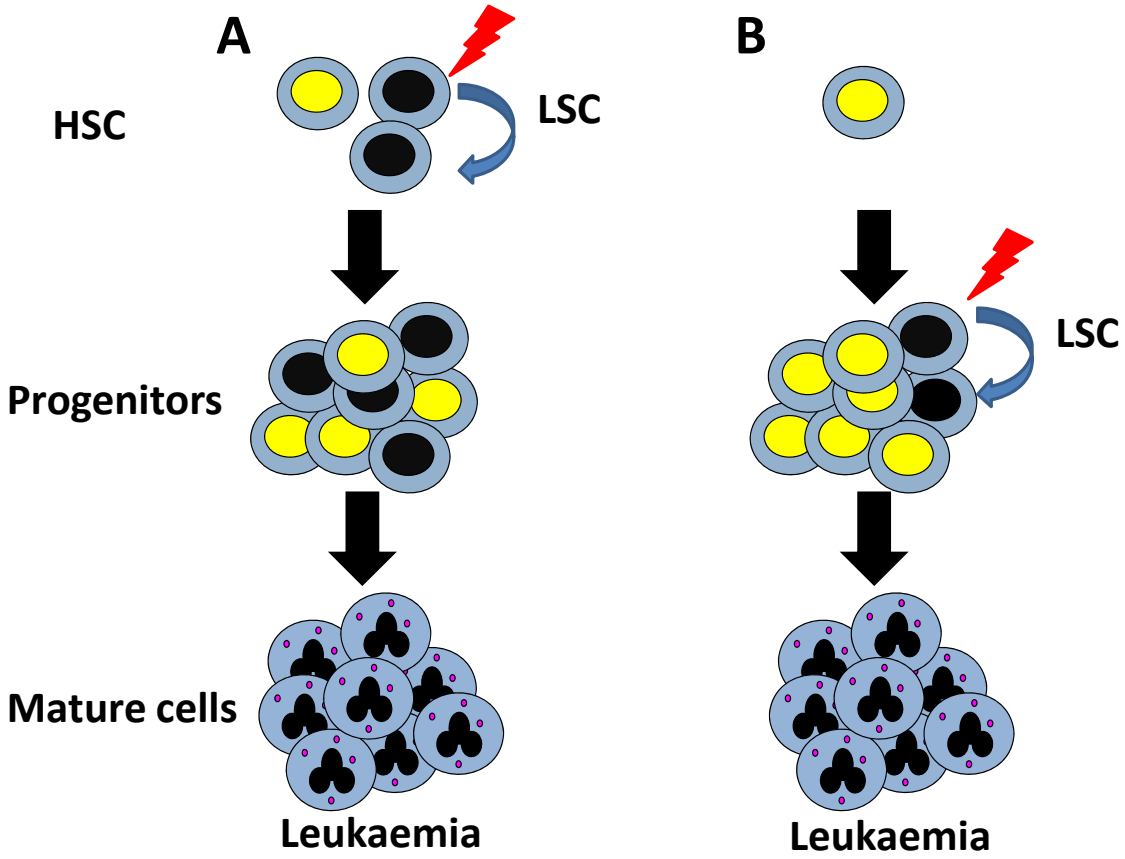


Figure 1-9 Models of LSC origin
The LSC can arise from the normal HSC compartment when the initial transforming mutation happens in a normal HSC (A). Alternatively the transforming mutation can arise in a progenitor which as a result reacquires LSC properties (self-renewal and differentiation) (B). Note it is also possible for both events to happen sequentially during the course of the same disease

1.2.2 The cancer stem cell (CSC) model and its implications

Since the original description of the hierarchical structure of human AML by Bonnet and Dick, a similar organisation has also been demonstrated in many other forms of leukaemias and solid tumours leading to the development of a new model to explain tumour heterogeneity which is applicable in all forms of tumours where a stem cell can be identified[173]. According to this model, usually defined as the CSC model, the CSC is the only cell within a specific tumour with the ability to self-renew and reconstitute the entire tumour (the same two key properties of normal HSC). Therefore CSC is biologically distinct from the bulk tumour cells and tumour heterogeneity is a result of the differentiation of CSC into tumour bulk cells. This model is often mentioned in contrast with another model for tumour heterogeneity, first described by Nowell in 1976 and called the clonal evolution model, which postulates that all cancers arise from a single cell of origin which will evolve through the progressive acquisition of genetic mutations within the original clone leading to the sequential selection of more aggressive subclones[174]. According to this model the heterogeneity of cancers is secondary to the presence at any one time of various genetically different subclones all equally capable of maintaining the tumour.

As is often the case, neither of the two models is probably completely correct. While the existence of CSC has been proven beyond doubt in several forms of leukaemias and solid tumours, clonal evolution is still observed within cancers following a CSC model. It is therefore now accepted that both the CSC model and the clonal evolution model can coexist, as in fact CSC can undergo clonal evolution during the course of a disease[175] possibly as a result of additional hits. As a consequence it is possible that several CSC with different properties might be present at any one time within a cancer. Nevertheless the CSC model is still very attractive because it helps to explain poorly understood clinical phenomena such as resistance to therapy, disease persistence, and relapse in the face of good initial response, especially in the absence of any evidence of clonal evolution within the resistant population of cells. In this latter scenario the CSC are in fact thought to be the resistant cells, potentially causing disease relapse, because of their intrinsic (possibly epigenetic) differences compared to the bulk of differentiated tumour cells[176]. Several lines of evidence support this hypothesis in various forms of leukaemias and solid tumours. For example a very recent study in the glioblastoma model used lineage tracing of stem cells to show *in vivo* that CSC exist and contribute to repopulating the tumour following standard cytotoxic treatment which only targets the bulk of the disease. Moreover using a

clever and elegant combination of "suicide gene technology" that selectively killed glioblastoma CSC together with standard cytotoxic treatment, the authors were able to show that targeting both CSC and their daughter cells prevented the growth of glioblastoma *in vivo*[177]. In conclusion, based on the CSC hypothesis, the main focus of the ongoing research in several forms of leukaemias (and in other forms of cancers following the CSC model) is to improve the identification as well as the functional and molecular characterisation of the LSC. This in turn should help to identify therapeutically exploitable differences between the LSC and their normal counterpart and lead to development of more effective therapies against them which, according to the original hypothesis, would lead to disease cure by directly targeting the "beating heart" of the tumour[173] (figure 1-10).

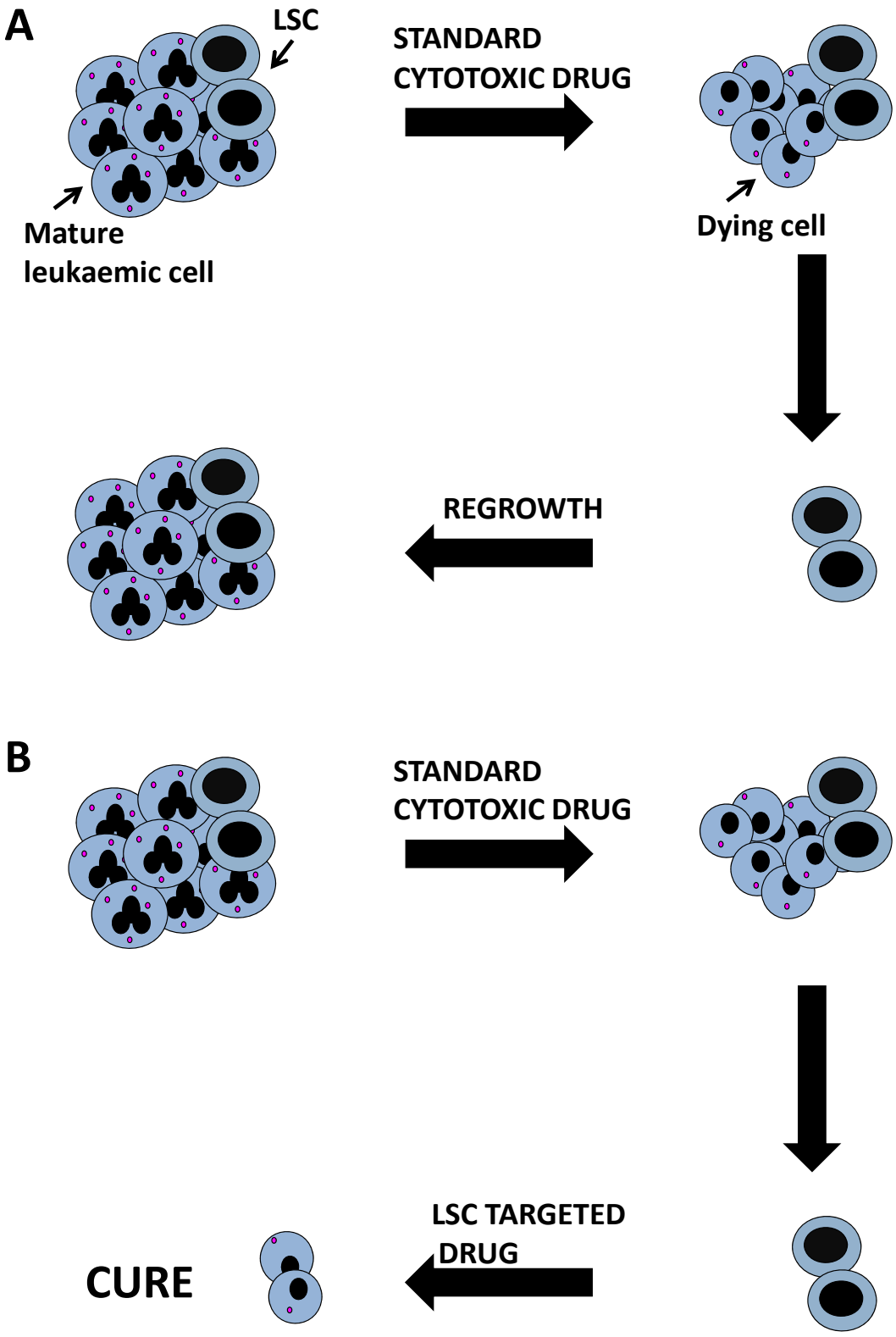


Figure 1-10 Model of disease relapse secondary to persistence of LSC and possible pathway to cure
Standard cytotoxic treatment is effective against mature leukaemic cells but spares LSC which can then repopulate the tumour (A). By combining a standard cytotoxic drug with a LSC targeted drug it would be theoretically possible to eliminate both mature leukaemic cells and LSC thus leading to disease eradication (B)

1.3 CML

1.3.1 Clinical features

CML is an acquired clonal myeloproliferative disorder resulting from the malignant transformation of a HSC. CML accounts for about 15% of all adult leukaemias and most commonly occurs in adults between the ages of 40 and 60, with a median age at presentation of 53 years. It normally runs a triphasic course from the initial chronic phase (CP) to an accelerated phase (AP) and then to blast crisis (BC), with the duration of each phase being highly variable between patients, but progressively shorter. In CP the disease is characterised by expansion of the myeloid compartment in the BM with resulting accumulation of mature neutrophils in peripheral blood and often also the presence of basophilia and thrombocytosis. Splenomegaly is often detected at this stage. If not treated CP CML lasts on average between 3 to 5 years before it progresses through an AP to BC stage, when a block in differentiation of the leukaemic SPC arises and leukaemic blasts accumulate in the BM and peripheral blood leading to a clinical picture similar to the one seen in AML characterised by progressive and severe BM failure with a poor prognosis[178,179].

1.3.2 The *BCR-ABL* oncogene and its role in CML development

Perhaps the most remarkable aspect in the history of CML is that it has been the first example of a cancer linked to a single chromosomal abnormality, later also shown to be disease causative. In 1960, an acrocentric chromosome, later shown to be a shortened chromosome 22, was described in all patients with CML by Nowell and Hungerford working in Philadelphia[180]. This chromosome has since been known as the Philadelphia (Ph) chromosome. In 1973, Rowley showed that the Ph chromosome was the product of a balanced translocation between the long arms of chromosomes 9 and 22[181]. Subsequently it was demonstrated that in CML, the oncogene *c-ABL*, normally present on chromosome 9, was translocated to the Ph chromosome, adjacent to the breakpoint cluster region, BCR[182] and this resulted in the generation of a fused transcript which was called *BCR-ABL*[183] (figure 1-11).

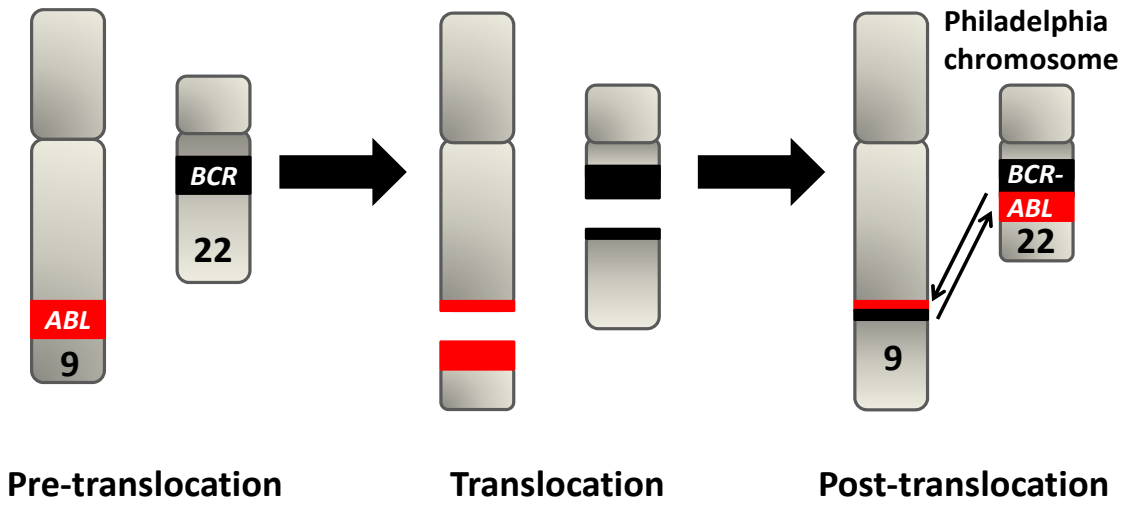


Figure 1-11 The Ph chromosome and *BCR-ABL* fusion transcript
The reciprocal translocation between chromosome 9 and 22 results in the formation of the Ph chromosome (shortened 22) and the *BCR-ABL* fusion transcript.

This transcript is in turn translated typically into a 210kDa protein (p210 BCR-ABL)[184], which exhibits constitutively elevated TK activity and can transform cells[185,186]. Further confirmation of the causative role of BCR-ABL in CML development *in vivo* was provided when murine BM cells transduced with a retrovirus encoding p210 BCR-ABL were shown to be capable of producing a CML-like disease when transplanted into an irradiated host[187]. More recently this has been confirmed in a transgenic mouse model where expression of BCR-ABL can be specifically induced in the HSC upon withdrawal of tetracycline from the drinking water. Consistent with the retroviral transduction model, these mice also developed a CML-like disease when BCR-ABL was induced in the HSC[188]. These experiments proved that BCR-ABL was responsible for inducing CML in a murine mouse model. Apart from its causative role in CML, it is also worth noting that the consistent presence of the specific BCR-ABL translocation in all CML patients has also allowed diagnosis and monitoring of disease response to treatment very effectively using both cytogenetic analysis of metaphases of BM MNC looking for the presence of the Ph chromosome and the ultrasensitive quantitative real time-polymerase chain reaction (qRT-PCR) to detect *BCR-ABL* transcripts in peripheral blood MNC[189]. As CML progresses to more advanced phases, the Ph chromosome is often no longer the single chromosomal abnormality as new cytogenetic abnormalities can be detected[178,179].

1.3.3 CML as a paradigm of the CSC model

CML is a form of leukaemia which perfectly fits the CSC model. Early experiments using patterns of inactivation in X-linked genes were able to show that CML was a clonal

disorder whose origin could be traced back to at least a multipotent stem cell as the same clone was detected in granulocytes, erythrocytes, platelets and monocytes[190,191]. The hierarchical organisation of CML, at least in its CP, also lends further support to this. In 1999, however, Holyoake *et al* provided formal demonstration of the existence of a CML LSC using standard transplantation experiments and *in vitro* assays[192]. The CML LSC reside in the $CD34^+$ $CD38^-$ compartment[193] and in fact it has since been shown in murine models that the BCR-ABL oncogene, in contrast with other known oncogenes, is only capable of driving leukaemia development when transduced into HSC which possess inherent self-renewal capacity, but fails to do so if transduced into more mature progenitor cells which have lost self-renewal potential[168]. When these findings are extrapolated to the disease in humans, they support a model whereby CML is initiated when the Ph chromosome arises in a single HSC at which point this is transformed into a CML LSC. The constitutive TK activity of the BCR-ABL oncoprotein in the CML LSC stimulates several signalling pathways which determine enhanced survival, proliferation and differentiation of the leukaemic clone compared to the normal haemopoietic cells in the patient BM. This in turn leads, over time, to expansion of the myeloid compartment with preserved cell maturation to terminally differentiated forms and appearance of clinical disease. If not treated, the presence and activity of BCR-ABL drive further genomic instability [194], and can lead to additional genetic changes or hits resulting in the progression of the disease from its CP to the AP and eventually to BC[195]. Once in BC stage it has also been shown that cells with the phenotype of a progenitor ($CD34^+$ $CD38^+$) acquire self-renewal potential and can act as LSC[196].

1.3.4 BCR-ABL structure and function

The human *ABL* gene encodes a 145kDa non-receptor TK expressed in most tissues. It is composed of several different domains. The amino terminal of ABL contains the SRC homology (SH) domains SH1, SH2 and SH3. SH1 is the TK functional domain, SH2 is mainly important for protein-protein interactions while the SH3 domain has a TK inhibitory function and plays an important role in regulating ABL TK activity. The centre of the protein contains proline-rich sequences which are also important in protein-protein interaction. The carboxyl terminal region contains domains important to protein localisation such as nuclear localisation signals, DNA and actin binding domains (figure 1-12 A). The ABL protein is normally present in both nucleus and cytoplasm of cells and its main function are to integrate signals from both intracellular and extracellular sources to effect cell fate decisions such as survival, proliferation and apoptosis. The exact functions

of ABL however are still not completely clarified and appear to be partly dependent on its localisation. Nuclear ABL has been shown to have a role in the regulation of cell cycle with mainly inhibitory function in the G1 phase and even induction of apoptosis. Its kinase activity appears to be stimulated by ionising radiation thus suggesting a role in cell cycle arrest and apoptosis in genotoxic stress. Less is known with regards to the function of ABL in the cytoplasm where it is found bound to F-actin[197,198]. However it is worth noting that all the transforming ABL proteins are exclusively localised in the cytoplasm thus suggesting that the transforming activities of this protein can only occur in the cytoplasm[199].

The 160kDa protein BCR is also a signalling protein ubiquitously expressed and containing multiple domains. The amino terminal contains a serine-threonine kinase and a coiled-coil domain necessary for its dimerisation. The centre and carboxy terminal of the protein contain respectively the pleckstrin-homology domain and a RAC guanosine triphosphatase activating protein (RAC-GAP) domain (figure 1-12 B). It is important to note that BCR itself can be phosphorylated on several tyrosine residues and in particular the tyrosine 177, by binding an adaptor protein called GRB2, plays an important role in RAS pathway activation[178,197,200].

As already mentioned the translocation of the 3' portion of ABL in proximity to the 5' portion of BCR leads to the formation of a fused *BCR-ABL* transcript which gives rise to the chimeric BCR-ABL protein. Three different breakpoints within the BCR sequence have been identified and depending on where the break occurs, three chimeric BCR-ABL proteins of different molecular weight can be produced (p190, p210 and p230 BCR-ABL). Most CML patients harbour the p210 BCR-ABL protein, while the p190 BCR-ABL is mainly seen in patients with Ph positive acute lymphoblastic leukaemia and rare patients with CML. p230 BCR-ABL has been associated with a variant form of CML, the so called neutrophilic CML, which tends to run a more indolent course, although classical cases of CML in association with p230 BCR-ABL have also been described in few instances[200-203]. However all three proteins, although demonstrating different levels of kinase activity, have been shown to have *in vitro* transformation properties and *in vivo* leukaemogenic activity[204]. The fusion of BCR to ABL enhances the protein TK activity possibly as a result of the abrogation of the suppressive effects of the ABL SH3 domain on the SH1 domain TK function and promotes its cytoplasmic localisation and dimerisation[197,200]. Through both autophosphorylation and substrate phosphorylation, BCR-ABL can directly and indirectly activate the signalling pathways central to its transforming activity[200,205].

In p210 BCR-ABL the fusion point occurs after the pleckstrin homology domain of BCR and before the SH domains of ABL, thus including the majority of the BCR domains and effectively all ABL domains. Therefore BCR-ABL is a relatively big protein with multiple domains (figure 1-12 C). Functional *in vitro* and *in vivo* analysis of the transforming activity of several mutated forms of BCR-ABL have shown that in addition to the crucial TK (SH1) domain, other domains and even single amino acid residues are essential to BCR-ABL transforming activity therefore supporting a model where the TK activity of BCR-ABL is necessary, but not sufficient on its own to induce CML[206]. The domains or residues shown so far to be crucial to BCR-ABL induced leukaemogenesis are together with the SH1 domain[207], the SH2[208,209] domain in the ABL protein, and the coiled-coil domain and the tyrosine 177 in BCR[210-212]. While SH2 and coiled-coil domain mutations reduce the TK activity of BCR-ABL, the tyrosine 177 mutant displays normal TK activity thus suggesting that it plays a role in BCR-ABL leukaemogenesis independently of BCR-ABL TK activity. The current knowledge of the signalling pathways activated by BCR-ABL through all its domains and of their effects on the biological properties of BCR-ABL positive cells and their leukaemogenic activity will be the subject of the next section. However it is worth pointing out now that a clear understanding of BCR-ABL induced signalling pathways has also highly relevant therapeutic implications. In fact, as it will be explained later in the Introduction, pharmacological targeting of the TK activity of BCR-ABL has become the mainstay of CML treatment. Understanding therefore which signalling pathways link to the domains crucial to BCR-ABL leukaemogenic activity, in particular for those domains or residues shown to be essential despite having no effect on TK activity, could identify together with novel mechanisms central to CML biology, novel therapeutic targets.

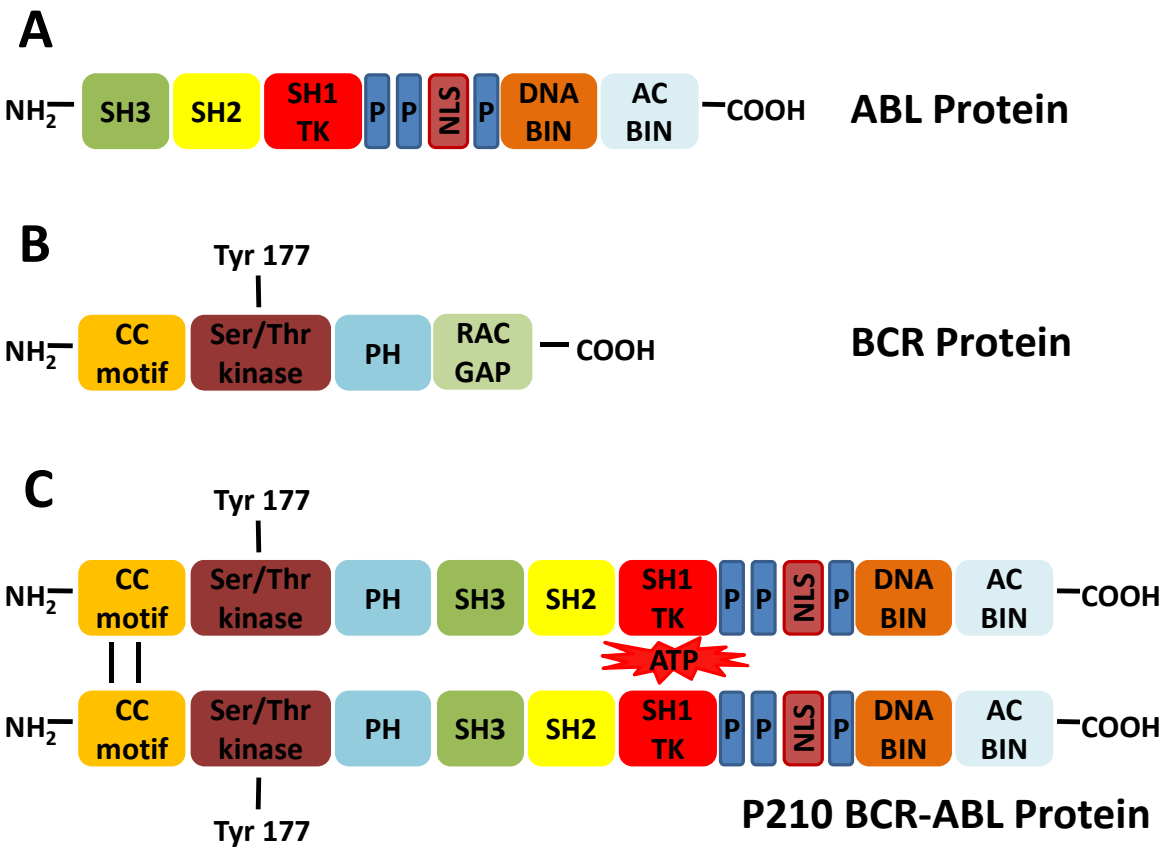


Figure 1-12 Structure of ABL, BCR and p210 BCR-ABL
ABL protein has three SRC homology (SH) domains in the amino terminal end with one of them having TK activity. Proline rich domain (P) and a nuclear localisation signal domain (NLS) are in the centre of the protein while a DNA binding (DNA BIN) and actin binding (AC BIN) domain are in the carboxy terminal. BCR has a coiled-coil (CC) and a serine/threonine (Ser/Thr) kinase domain in the amino terminal. A pleckstrin homology (PH) domain is in the middle and a RAC-GAP domain in the carboxy terminal. The tyrosine 177 (Tyr 177) in BCR, by binding an adaptor protein called GRB2, plays an important role in RAS pathway activation. p210 BCR-ABL arises from the fusion of BCR downstream of PH domain with ABL just before the SH3 domain. p210 BCR-ABL can form dimers through the CC domain (black lines) and has enhanced TK activity (indicated by the ATP molecule sitting in the pocket of its TK domain).

1.3.5 Signalling pathways activated by BCR-ABL and the mechanisms of BCR-ABL induced transformation

The main mechanisms of BCR-ABL induced transformation are enhanced proliferation, reduced apoptosis and abnormal interaction with BM stroma. These features are a result of the activation of several signalling pathways which relay both mitogenic/proliferative and antiapoptotic signals. Some of these pathways overlap with pathways activated following GF stimulation of normal HSPC[213-215]. Their direct activation by BCR-ABL therefore explains the proliferative phenotype of BCR-ABL driven leukaemias and their GF independence *in vitro*[216]. BCR-ABL activates these pathways both by direct phosphorylation of substrates via its TK activity or indirectly following its autophosphorylation. The latter produces increased phosphotyrosine sites on the BCR-ABL protein which can act as docking sites for proteins carrying SH2 domain (able to bind to phosphotyrosine). These proteins can then either act as adapters for other proteins with catalytic function or they have their own catalytic function. The eventual outcome however is the activation of some known mitogenic and antiapoptotic pathways and amongst those the RAS/MAP kinase, JAK/STAT, PI3 kinase/AKT and NFκB pathways have all been shown to be activated by BCR-ABL and to be crucial to its leukaemogenic activity[200,217].

1.3.5.1 BCR-ABL and the RAS/MAP kinase pathway

In CML RAS is constitutively active at least in part as a result of BCR-ABL autophosphorylation on tyrosine 177[218]. This phosphotyrosine site in fact acts as a binding site for the adaptor protein GRB2 which binds and activates a guanine exchange factor protein called SOS leading to stabilisation of RAS in its active form[200]. Similarly GRB2 mutants also block RAS activation and BCR-ABL transforming capability[219]. Also other adapter proteins such as SHC and CrKL have been shown to be involved in RAS activation although the exact mechanism is less clear[220-222]. In the same reports it was shown that activation of RAS/MAP kinase pathway has a central role in BCR-ABL transforming ability both *in vitro* and *in vivo* although this varied slightly according to the cell context[218,220-222]. Co-transfection of a RAS dominant negative mutant in BCR-ABL transfected cells was also able to inhibit BCR-ABL induced transformation[223]. Both genes involved in cell cycle progression, such as cyclin dependent kinases, leading to G1 to S phase transition and DNA synthesis[224] or mediating reduced apoptosis of the leukaemic cells, such as the antiapoptotic proteins BCL-2[225], have been shown to be activated by the RAS pathway in BCR-ABL positive cells. Taken together this evidence suggests that

RAS/MAP kinase pathway is constitutively activated and plays a central role in BCR-ABL induced leukaemogenesis through upregulation of antiapoptotic and proliferative signals.

1.3.5.2 BCR-ABL and the JAK/STAT pathway

In BCR-ABL positive cells constitutive activation of several members of the JAK/STAT pathway has been shown, including JAK1/2/3 and STAT1/3/5[226-230]. Several domains of BCR-ABL have been shown to be necessary to the activation of both JAK and STAT with the TK activity and SH2 domain important for JAK2 activation[231] , the SH2 and SH3 and TK activity domain important for STAT5 activation[232] and the tyrosine 177 (via activation of the RAS pathway) and TK activity domain for STAT3 activation[229]. Amongst the JAK family members, JAK2 appears to play a central role in BCR-ABL induced leukaemogenesis, at least in its advanced phase, as kinase inactive JAK2 mutant shows reduced clonogenic potential and ability to form tumours in nude mice[231]. Amongst all the STAT family members, STAT5 has a central role in BCR-ABL induced transformation as shown by the inhibitory effects of dominant negative forms of STAT5 on BCR-ABL induced transformation *in vitro*[232-234]. Initial *in vivo* experiments using an amino terminal deleted form of STAT5 underestimated the role of STAT5 in BCR-ABL induced leukaemogenesis[235]. However recently its central role to BCR-ABL induced leukaemogenesis has been confirmed *in vivo* using complete *Stat5* KO mouse BM cells transduced with *BCR-ABL* retrovirus[236]. STAT5 contribution to BCR-ABL induced transformation is also via activation of both genes involved in cell proliferation (*cyclin D1*)[237] and against apoptosis (*BCL-* X_L)[238].

A controversial point in the CML community is the relative contribution of JAK2 and BCR-ABL to the activation of STAT5 in CML cells. While in normal haemopoiesis the role of JAK2 in activating STAT5 has been demonstrated, in BCR-ABL positive cells a dominant negative JAK2 mutant was unable to prevent BCR-ABL induced STAT5 activation[230]. More recently the dispensable role of JAK2 in both BCR-ABL induced leukaemogenesis and STAT5 activation has also been shown *in vivo* using *Jak2* KO mouse BM cells transduced with *BCR-ABL* retrovirus[239]. Conversely JAK2 appears to be able to induce MYC expression in BCR-ABL positive cell lines in a STAT5 independent fashion[240] and as already mentioned has been shown to have a role in BCR-ABL induced transformation[231]. Therefore it appears that the presence of BCR-ABL perturbs the normal activation of the JAK2/STAT5 pathway with at least a partial uncoupling of the signals relayed by these two proteins. As investigations on the role of JAK2 are a relevant part of

the work presented in this thesis, this issue will be analysed in more detail in one of the results chapters.

1.3.5.3 BCR-ABL and the PI3 kinase/AKT pathway

The PI3 kinase/AKT pathway is another pathway which has been shown to be required for BCR-ABL positive cells survival and proliferation[241]. Similar to the RAS/MAP kinase and JAK/STAT pathway, it prominently induces mitogenic signals and reduces proapoptotic ones. In BCR-ABL positive cells, PI3 kinase specifically activates AKT kinase and the BCR-ABL SH2 domain appears to have a central role in this[242]. This activation is not secondary to direct interaction of PI3 kinase with BCR-ABL[243], but requires the presence of the adaptor protein complexes, such as GRB2/GAB2 and CrKL/CBL which respectively bind to BCR-ABL residues[244,245](i.e. tyrosine 177) or domains[246] and provide docking sites for PI3 kinase. The activation of the PI3 kinase/AKT pathway is essential for BCR-ABL induced leukaemogenesis as demonstrated by the ability of a dominant negative AKT mutant to inhibit BCR-ABL dependent transformation of murine BM cells *in vitro* and leukaemia development in immunodeficient mice. PI3 kinase/AKT pathway transforming ability appears to be at least partially secondary to the increased expression and transcriptional activation function of MYC[242]. MYC is a transcription factor shown to be active in several malignancies and also shown to be necessary to BCR-ABL induced transformation[247]. Moreover the activation of the PI3 kinase/AKT in BCR-ABL positive cells leads to phosphorylation and inactivation of the same substrates as in their BCR-ABL negative counterparts, including BAD[248], CDKN1B/p27[249] and the FOXO transcription factors[250]. Inactivation of the FOXO transcription factors by BCR-ABL also has similar effects on the expression levels of its target genes, such as *BIM*[251] and *D cyclins*[88] as those observed in other cell types following GFs stimulation.

1.3.5.4 BCR-ABL and the NFκB pathway

NFκB has been shown to play a role in a variety of solid and haematological malignancies[150]. In CML, constitutive activation of NFκB has been demonstrated in BCR-ABL positive cells[252]. Moreover using *in vivo* and *in vitro* experiments, its role in BCR-ABL induced leukaemogenesis and transformation of BM cells has been shown[253]. Indirect evidence for its importance in survival of CML cells including primary samples from CML patients has been provided by the ability of NFκB pathway inhibitors, such as IKK inhibitors, to induce apoptosis of CML cells alone and in combination with BCR-ABL tyrosine kinase inhibitors (TKI)[254,255]. BCR-ABL TK activity has been shown to be

essential[253,256], while the tyrosine 177 has been shown to be dispensable[256] for NFκB activation. However the exact role of NFκB in BCR-ABL induced leukaemogenesis, survival and proliferation is still unclear. Only recently a potential role of NFκB in preventing accumulation of excessive levels of ROS in BCR-ABL positive cells has been suggested as a possible mechanism for its protective role in this cell context[257].

1.3.6 The role of GFs in survival, proliferation and maintenance of CML cells

The role of GFs in CML cell survival, proliferation and maintenance is an area of active research but also of contention. The known ability of BCR-ABL oncoprotein to activate the same signalling pathways normally activated by GFs, coupled with GF independence *in vitro* [216]of CML cells has led many investigators to question the exact role of GFs in CML pathology and if indeed the GFs signals are dispensable in the context of a BCR-ABL transformed cell. In this next section therefore a review of the current evidence on the role of GFs in CML induction, expansion and maintenance will be presented. Moreover the aberrant autocrine GFs production in CML cells and the effects of the presence of BCR-ABL oncoprotein on the BM microenvironment and the normal physiological responses to GFs in CML cells will be also reviewed.

1.3.6.1 Exogenous GFs role in CML cells

Several studies have shown that CML cells respond normally to exogenous GFs. This has been shown for both proliferative GFs important for myeloid differentiation, such as IL-3, GM-CSF and G-CSF, and negative regulators of proliferation, such as TGF-β1, mainly through their effects on cell survival and proliferation[258,259]. Since the introduction of BCR-ABL TKI it has also been possible to show more easily the ability of several of the proliferative GFs mentioned above to activate their normal signalling pathways upon BCR-ABL kinase inhibition with sometimes even a compensatory upregulation of the same pathways (MAP kinase) observed[260-263]. Moreover this has also allowed the demonstration that GFs signalling could provide survival cues to CML cells when BCR-ABL kinase is inhibited and that therefore this could be a resistance mechanism to current TKI based therapies. In fact by targeting these pathways, such MAP kinase[261], PI3 kinase/AKT[264], JAK/STAT[260,262] and NFκB[254] through specific small molecule inhibitors, an increased kill of BCR-ABL positive cell lines and also CML primary $CD34^+$ cells was demonstrated.

The role of exogenous regulators of normal HSC maintenance has also been studied in CML LSC maintenance. In order to prove their importance in most cases a standard retroviral transduction/transplantation model has been used where BM cells carrying a deletion for an important component of these signalling pathways were transduced with *BCR-ABL* retrovirus and the effects on CML development in the mice were observed, and if possible, this was complemented using inhibitor work *in vivo*. This has provided proof of a role for many such regulators like Hh[104,265] and Wnt/β-catenin[266-268] and TGF-β1[111]. In the case of TGF-β1, it was also shown that the mechanism involved activation of FOXO through inhibition of AKT in a very similar fashion to what had also been observed in normal HSC.

In conclusion these studies show that similar cues involved in normal HSPC maintenance, survival and proliferation are also active in the CML counterparts, thus proving their responsiveness to exogenous GFs and that CML cells appear to have an intact and functional response mechanism to cytokines despite the presence of BCR-ABL oncoprotein. The GFs signals appear also to play a central role in maintenance of disease and as survival cues particularly in the presence of TKI.

1.3.6.2 The autocrine production of GFs in CML cells

Together with the normal response to exogenous GFs, CML cells also display aberrant autocrine production of several GFs. Following the original demonstration of an autocrine loop in BCR-ABL positive cell lines for GM-CSF and IL-3[269], a similar observation was later made in primary CML cells too. This autocrine loop resulted in STAT5 activation and GFs independent growth of CML $CD34^+$ cells, both of which could be inhibited using neutralising antibody[270]. These studies further confirmed that despite the presence of BCR-ABL, CML cells are responsive to GFs stimulation and in fact enhanced autocrine GFs signalling could even be relevant to BCR-ABL induced transformation. Since then autocrine loops with a similar role in cell survival and proliferation have also been demonstrated in BCR-ABL positive cell lines and CML primary MNC for other GFs, such as IL-4[271] and GM-CSF[262]. In order to further confirm the role of autocrine GF production in BCR-ABL induced leukaemogenesis, *in vivo* models have been used with some conflicting results. BM cells derived from *Il-3* plus *Gm-csf* KO mice and transduced with *BCR-ABL* were able to induce a BCR-ABL induced myeloproliferative disorder identical to that induced by *Il-3* plus *Gm-csf* expressing BM into syngeneic animals. This was true regardless of the genotype of the recipient, i.e. a CML disease was induced in both *Il-3*

plus *Gm-csf* KO and genetically normal recipient[272]. This result supported a model where IL-3 and GM-CSF are redundant for the generation of CML. However it should be noted that although commonly used, the BCR-ABL retroviral model does not fully reflect the clonal disorder with the long latency period that gives rise to CML in humans and the high levels of expression of BCR-ABL in this model (higher than in primary CML cells) could render GFs role dispensable. In fact, when *Il-3* KO BM cells transduced with *BCR-ABL* were cultured *in vitro* for an extended period, they lacked leukaemogenic activity in transplanted mice in comparison to *Il-3* expressing counterparts[273]. These latter observations were more supportive of a model whereby the maintenance of transplantable CML LSCs in culture requires autocrine IL-3, thus implying that autocrine IL-3 is indeed relevant to the sustained leukaemogenicity of CML LSCs in a situation more closely approximating that seen in the human disease. Moreover the protective effects of GFs might become more apparent in the context of an inhibited BCR-ABL kinase as previously mentioned with regards to CML cells response to exogenous GFs. However in neither of these two studies were the mice challenged with TKI and this might have masked an even more important effect of autocrine GFs in CML pathogenesis.

1.3.6.3 Aberrant GF responses in CML cells

Another important point to consider when assessing the role of GFs in CML biology is the effects that BCR-ABL presence has on CML cells responses to GFs and overall BM microenvironment. For example it has been observed that in BCR-ABL transduced haemopoietic cell lines, the BCR-ABL oncoprotein directly interacts with the IL-3/GM-CSF β chain receptor, inducing activation of GF signalling in a ligand independent way and causing therefore an aberrant constitutive activation of GF signalling via its normal receptors[274]. Conversely it has been reported that soluble factors, such as monocyte chemotactic protein 1 (MCP-1), selectively inhibit the proliferation of normal progenitors *in vitro* while sparing CML progenitors[259]. The work of Bhatia *et al* in both primary CML samples and CML transgenic mouse models has been particularly helpful in furthering the understanding of the effects BCR-ABL has on the BM microenvironment. In original work carried out with primary CML and normal BM samples, it was shown that indeed BM stroma from CML patients had a reduced ability to support growth of normal LTC-IC. This appeared to be secondary to the presence of malignant stromal macrophages in CML stroma rather than abnormal production of soluble factors as demonstrated by the similar concentration of the cytokines tested in BM supernatant from CML and normal BM. However potential indirect effects secondary to altered function of CML stromal cells in

binding and presenting haemopoietic GFs to CML and normal HSPC could not be excluded[275].

In the last year, work from the same group has also better characterised the BM microenvironment in CML using mainly a transgenic BCR-ABL mouse model. This work has shown that indeed the levels of some cytokines are different between CML and normal BM with higher levels amongst others of IL-1, IL-6, G-CSF and TNF-α in the former. Moreover this altered GF milieu determined a reduced engraftment of normal HSC compared to CML LSC when these where transplanted in BCR-ABL expressing irradiated host. The ability of normal HSCs to grow in the presence of BM plasma from CML mice was also impaired while CML LSCs were unaffected. Higher levels of *IL-1*, *G-CSF* and *TNF-α* mRNA levels were also demonstrated in BM MNC of CML patients compared to normal BM MNC. Again in contrast to human CML LSCs, human HSCs showed reduced growth in the presence of CML BM conditioned media[276]. Together these results suggest that altered BM microenvironment in CML confers a growth advantage to CML LSCs compared to normal HSCs. These effects are secondary to both the presence of soluble factors, such as G-CSF, TNF-α and IL-1, and direct interaction with stromal cells. Moreover although in certain cases, as shown previously, CML cell responses to GFs appear similar to what is observed in normal HSPC, in other instances CML cells show aberrant responses to these environmental cues which they can use to their own avail.

1.3.7 Current therapies and clinical problems in the management of CP CML

The treatment of CML has to be considered in the field of medicine one of the success stories of the past 50 years. Since the original description of the Ph chromosome through the demonstration of the pathogenic role of the *BCR-ABL* oncogene to the clinical development of specific inhibitors of BCR-ABL kinase activity, CML has served as a paradigm of how a clear understanding of the molecular cause of a disease can lead to the rational development of specific targeted therapies with high therapeutic index and massive impact on disease outcome.

1.3.7.1 Historical perspective

Before the molecular basis of CML was completely clarified, its treatment was based, as is still the case these days for many other cancers, on standard cytotoxic chemotherapy such as alkylating agents like busulphan or the ribonucleotide reductase inhibitor, hydroxyurea.

Although capable of inducing haematological responses, none of these treatments were capable of inducing cytogenetic responses, i.e. reduction or disappearance of Ph^+ cells in the BM of patients (see table 1-4 for definition of responses in CML patients) and therefore they did not alter patient survival. Moreover in the case of busulphan, significant and severe associated side effects, mainly in the form of myelosuppression and pulmonary fibrosis, were also present[277]. The introduction of IFN-α in the 80s provided the first significant improvement in patient life expectancy (15% higher 5 year survival rate compared to chemotherapy in a meta-analysis of seven randomised trials). Improved survival in patients treated with IFN-α was then shown to be associated with achievement of a cytogenetic response (at least major) which has since been used as a surrogate marker for patient survival[278,279]. Both survival and cytogenetic responses could be improved when IFN-α was combined with the antimetabolite, cytarabine[280]. However IFN-α parenteral administration and poor tolerability (such as "flu like" symptoms, lethargy, insomnia and depression) made this treatment still relatively difficult to deliver. At the same time when IFN-α was gaining support in CML treatment, allogeneic BM transplant (alloBMT) also started to be used for CML patients. Unlike any other treatment before (and probably after), alloBMT offered curative potential for patients and in that respect represented a significant improvement compared to any other therapies available. However its treatment morbidity and mortality rate, which, depending on several variables, can be as high as 40%, limited its use to a small cohort of patients (20-25% of patients), generally young with a suitable donor and no other significant comorbidity[281].

The demonstration at the beginning of the 90s that the BCR-ABL oncoprotein was disease causative mainly because of its constitutive TK activity[185-187] led to the testing of the hypothesis that pharmacological inhibition of the BCR-ABL TK activity via small molecule inhibitor could be clinically useful.

Response by type	Definitions
Haematological	
Complete (CHR)	White blood cells <10X10^9/L Basophils <5% No myelocytes, promyelocytes, myeloblasts in the differential Platelet <450X10^9/L Spleen not palpable
Cytogenetic	
Complete (CCyR)	No Ph$^+$ metaphases
Partial (PCyR)	1% to 35% Ph$^+$ metaphases
Minor (mCyR)	36% to 65% Ph$^+$ metaphases
Minimal (minCyR)	66% to 95% Ph$^+$ metaphases
None (noCyR)	>95% Ph$^+$ metaphases
Molecular	
Complete (CMR$^{4.5}$)	Undetectable *BCR-ABL* mRNA transcripts by qRT-PCR in two consecutive samples of adequate sensitivity (the transcripts have to be undetectable in an assay with a sensitivity of at least ≥4.5 log)
Major (MMR)	Ratio of *BCR-ABL* to control gene ratio of ≤0.1% on an international scale

Table 1-4 Definition of haematological, cytogenetic and molecular responses in CML
Abbreviations CHR, complete haematological response; CCyR, complete cytogenetic response; PCyR, partial cytogenetic response; mCyR, minor cytogenetic response; minCyR, minimal cytogenetic response; noCyR, no cytogenetic response; CMR, complete molecular response; MMR, major molecular response. When CCyR and PCyR are counted together they are defined major cytogenetic response (MCyR). Ph$^+$ metaphases are examined in at least 20 BM MNC metaphases. For assessment of molecular responses, the conversion of each laboratory data to an international scale is recommended, to correct for the variability of the assays in different laboratories.

1.3.7.2 Imatinib mesylate

In 1996, Druker *et al* reported preclinical data showing that a modified 2-phenylaminopyrimidine (CGP 57148) induced apoptosis of BCR-ABL positive human cells, including primary CML cells with little toxicity on normal cells[282]. This compound, later known as imatinib (IM), specifically inhibits BCR-ABL TK activity in cellular tyrosine phosphorylation assays with a 50% inhibitory concentration (IC_{50}) of 0.25μM. Activity against the TK of the c-KIT receptor[283] and platelet derived growth factor receptor (PDGFR) were in similar ranges, but otherwise there was no other significant kinase inhibition close to that concentration range thus confirming its selectivity. IM functions as a competitive substrate for the BCR-ABL TK ATP binding pocket, thus preventing ATP from binding to the kinase to activate it (figure 1-13). IM reached clinical development as an oral agent in 1998 and rapidly showed in a large phase 3 clinical study (International Randomized Study of Interferon and STI571-IRIS) its ability to induce significantly higher

rates of cytogenetic responses and molecular responses, and therefore improve overall survival (OS), in newly diagnosed CML patients compared to the previous standard therapy based on IFN-α and cytarabine[284,285]. Since its introduction, IM has radically changed the management of CP CML and established itself as standard of care in newly diagnosed CML patients. The last follow-up reported for the IRIS study at 8 years shows continued clinical benefit with 83% of patients achieving CCyR, event free survival (EFS) rate of 81% and OS rate of 85%, which rises to 93% if CML only related deaths are considered, while also showing a safe toxicity profile[286]. Durability of responses was confirmed by the continually declining rate of progression events over time in patients who achieved CCyR on IM. Overall only 3% of patients achieving a CCyR progressed to AP/BC and all but one progressed within the first 2 years of achieving CCyR. These findings support the conclusion that once a patient achieves a sustained CCyR (at least 2 years), his/her chance of progressing are extremely low and recently an independent worldwide study reported that patients who achieve a stable CCyR on IM show an OS rate of 95.2% after 8 years, which is similar to the survival rate in the general population[287].

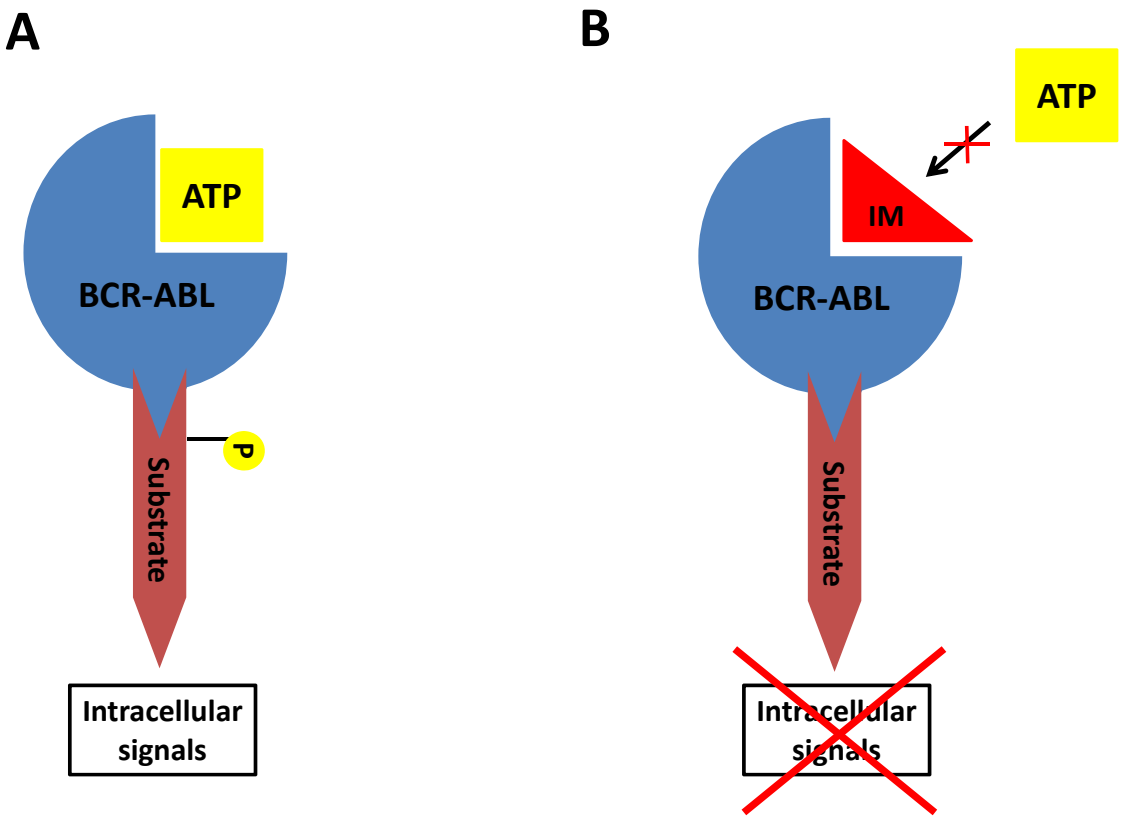


Figure 1-13 Mechanism of action of IM
BCR-ABL is able to phosphorylate its substrates and activate intracellular signals only when it binds ATP (A). IM competes with ATP for its binding site on BCR-ABL thus preventing activation of the oncoprotein and the downstream intracellular signals (B).

1.3.7.3 Disease resistance

Despite IM's remarkable efficacy, it is still felt however in the CML community that there is a genuine need for improving outcomes for CML patients. Analysis of response related prognostic factors from several studies of newly diagnosed CML patients treated with IM have shown that achieving a CCyR by 12 months is a consistent and reliable predictor of long-term outcome on IM therapy. Over 95% of patients who achieved this response did not progress to advanced disease by 5 years compared to only 75-80% of patients who failed to achieve a 12 months CCyR[288,289]. These results explain the previously described low risk of progression and normal life expectancy of patients achieving a sustained CCyR and have led investigators to consider a timely CCyR as the minimum satisfactory response for patients on IM[290]. The results reported in IRIS are known to suffer a selection bias as patients who discontinued IM for adverse events or failed to achieve a CCyR were censored and their outcome not considered in the IM efficacy analysis. In fact if the number of patients not achieving CCyR in IRIS (17%) is considered together with those who lost their response (about 10%), then a third of patients did not have an acceptable outcome on IM. In fact if a more stringent definition of EFS (which also includes not achieving a timely CCyR and adverse events as criteria for failing IM therapy) compared to the one applied in IRIS is used, lower EFS rates of 50-60% for newly diagnosed CP CML patients treated with upfront IM are reported as shown by three independent population based studies[289,291,292]. Even if the failures secondary to IM intolerance (which account for 10-20% of the total events) in the three above mentioned studies are not considered, these figures still show that about a third of CML patients treated with IM show evidence of resistance. More recently Marin *et al* has shown that a molecular response level at 3 months of less than 10% *BCR-ABL/ABL* transcripts is the strongest prognosticator in a multivariate analysis of long-term outcome in patients treated with IM and its prognostic value is independent of whether the patient had treatment discontinuation. Using these criteria, 24% of patients in the cohort studied were identified as being at high risk of progression thus confirming that resistance is a problem at least in about a quarter of patients treated with upfront IM[293]. All these data consistently show that together primary disease resistance (failure to achieve a satisfactory response) and secondary disease resistance (loss of a satisfactory response) are still significant clinical problems as they put all these patients at risk of disease progression for which current therapies are still unsatisfactory thus reducing their life expectancy.

1.3.7.3.1 Mechanisms of disease resistance and current therapeutic options

Although the mechanisms of IM resistance vary widely, they can be schematically divided in two main groups: BCR-ABL dependent and BCR-ABL independent.

The presence of both BCR-ABL kinase domain mutations and BCR-ABL gene amplification in CML samples from patients who had lost their response to IM were the first to be identified[294]. BCR-ABL kinase domain mutations are now the most common and best characterised resistance mechanism, especially in the cases of secondary resistance. Using a highly sensitive technique which can detect clones present at low levels, mutations are usually identified in over 50% of patients at disease relapse[295]. The original mutation identified in 6 of the 11 CML relapsed patients was a single nucleotide change resulting in a threonine to isoleucine substitution at position 315 (T315I) of ABL. These amino acid residues are present within the ATP binding site and the activation loop of ABL which are required for IM binding. As the threonine at position 315 is necessary to form a hydrogen bond with IM, its substitution results in loss of this interaction which, coupled with the steric hindrance from an extra hydrocarbon group on the isoleucine residue, results in the inhibition of IM binding[294]. To date the T315I mutation is still the most clinically relevant mutant because it has proven to be resistant to most available TKI. However many other mutations have now been identified with each of them having different consequences in terms of resistance conferred to the clone. Mutations normally affect IM binding either by direct inhibition through modification of an amino acid involved in drug binding to the kinase (as for T315I) or indirectly by altering BCR-ABL oncoprotein conformation[296]. Another BCR-ABL dependent resistance is reduced intracellular drug level due to either overexpression of multidrug efflux transporters of the ATP-binding cassette (ABC) transporter family, such as ABCB1[297], or reduced expression of the drug influx organic cation transporter OCT-1[298]. However for ABCB1, the experimental evidence supporting its role in resistance to IM is not clear. In fact conflicting results have been reported in BCR-ABL positive cell lines overexpressing ABCB1[299,300], while in the more relevant model of primary CP CML samples ABCB1 activity was low and its inhibition did not enhance IM induced BCR-ABL inhibition or kill[301]. Conversely more convincing data are in support of OCT-1 activity in IM resistance as high levels are predictive of better response to IM in clinical studies and inhibiting OCT-1 activity with its inhibitor prazosin resulted in reduced intracellular uptake of IM. Finally in patients with low OCT-1 activity, higher doses of IM were able to produce better responses[302].

All the above described resistance mechanisms confirmed that BCR-ABL oncoprotein is still a legitimate target in a proportion of resistant cases and this has led to the development of novel TKI of higher potency and binding affinity to BCR-ABL even in the presence of mutants. Nilotinib (NL) and dasatinib (DA) were the first two of these novel agents to be developed. NL is 10 to 20 fold more potent than IM in both phosphorylation and proliferation assays with IC_{50} in both types of assay in the nM range. NL is also quite specific with only PDGFR and KIT again inhibited at about 5 to 10 fold higher concentration than BCR-ABL[303]. Another advantage of NL is that its intracellular levels are not affected by drug transporters[304]. DA is an even more potent BCR-ABL inhibitor than NL with IC_{50} of less than 1nM. Moreover it also inhibits a number of SRC kinases at the same IC_{50} while retaining inhibitory activity against PDGFR and KIT too at low nM range[305]. Both compounds have shown activity against the majority of BCR-ABL mutants although mutants conferring resistance to either of them have been reported while the T315I mutant confers resistance to both[306,307]. Clinical studies of NL and DA in IM resistant/intolerant patients showed good efficacy with both agents producing CCyR in about 50% of patients[308,309]. The good efficacy of both NL and DA in the resistant setting have led to their use in newly diagnosed patients where they have both been shown to induce deeper responses more rapidly compared to IM. However longer follow-up is required to know if these faster responses will translate into reduced progression rates and improved survival[310,311].

More recently two other novel TKI have reached clinical development: bosutinib (BOS) and ponatinib (PON). BOS is a dual SRC/BCR-ABL inhibitor at low nM concentrations and, similarly to NL and DA, shows activity against multiple BCR-ABL mutants except T315I[312]. Conversely PON is the first TKI to show activity against T315I in experimental models with the ability to suppress the growth of all mutant BCR-ABL clones at 40nM in mutagenesis assays[313]. Its efficacy in this setting has now been confirmed in a recently published phase 1 clinical trial where PON has been shown to produce CCyR in about 75% of CP CML patients carrying a T315I mutation and in over 50% of multiresistant CP patients[314]. Summarising these data it is clear that novel TKI have been able to improve responses significantly in about half of resistant patients including T315I now thanks to PON. However another 50% of patients are still not being rescued. In those patients it is likely that BCR-ABL independent resistance mechanisms are present which means that CML cells rely on alternative survival pathways to support their survival and proliferation.

Several putative mechanisms have already been proposed for BCR-ABL independent resistance and amongst those the aforementioned GF activated signalling pathways (see 1.3.6.1) appear to definitely play a role. Another mechanism which has been well elucidated is the activation of the SRC kinases LYN and HCK in IM resistant samples. This has been demonstrated in both resistant BCR-ABL positive cell lines and primary samples from patients who lost response to IM and progressed to advanced phase disease. Moreover SRC inhibition induced apoptosis of these resistant cells[315,316]. These reports provided the rationale for the use of dual SRC/ABL inhibitors such as DA in advanced phase disease and DA is currently the only TKI licensed in this setting. More recently other pathways have also been involved in this type of resistance, such as the MAP kinase pathway through both constitutive activation of ERK kinases or paradoxical activation by TKI in the setting of resistant CML cells[317]. Again inhibition of ERK kinases was able to overcome this form of resistance in BCR-ABL positive cell lines[318,319]. However this approach has not yet been taken into clinical practice. It is possible that other novel resistance mechanisms will be elucidated in the future and that all will play a role to various extents in different groups of patients. The biggest challenge will be to translate these findings into clinical practice as precise understanding of the resistance mechanisms present in each patient will be required in order to devise an effective targeted therapy.

1.3.7.4 Disease persistence

For those patients who achieve satisfactory responses with TKI, the main problem is disease persistence. Although a small study on a subset of IRIS patients showed that up to 40% of IM treated patients achieved undetectable *BCR-ABL* transcripts levels at a median follow-up of 81 months[320], these figures have been much smaller (only 3%) in a large IM treated population based study with a median follow-up of 38 months, likely a reflection of the unselected group of patients in this population study and the shorter follow-up[289]. Therefore it is reasonable to say that only a minority of patients achieve sustained undetectable *BCR-ABL* transcripts. Moreover achieving undetectable *BCR-ABL* transcripts does not equate to disease eradication as shown by the detection of *BCR-ABL* genomic DNA in all patients who are negative by standard qRT-PCR for *BCR-ABL* transcripts in peripheral blood in two independent studies[321,322]. Even more importantly, if treatment is discontinued in this small cohort of patients with sustained undetectable *BCR-ABL* levels, approximately 60% will relapse at molecular level within 12 months, although they can all be rescued by reintroduction of therapy[323]. Overall these results prove that IM is not curative in the majority of patients, which results in an ongoing potential risk of disease

progression and need for lifelong treatment, which can be highly expensive and not readily accessible in many parts of the world. Moreover in those patients fortunate enough to have access to continuous treatment with IM, it has been shown that a small proportion will experience occasional severe side effects and the majority will suffer from mild/moderate continuous side effects perceived as affecting their quality of life and impairing their physical and social functioning. Interestingly these effects were more marked in the younger patients[287,324]. Continuous side effects, together with the chronic nature of the IM therapy, have been shown to affect patient's adherence to treatment and in fact about 30% of patients are not fully compliant to treatment at a median of 5 years from diagnosis, with only 14% of patients taking their medication exactly as instructed. Interestingly again younger patients were more likely to have lower adherence[325,326]. Lack of adherence to drug therapy has now been shown to correlate with disease response and in fact it might be the main reason for losing a CCyR in patients on long-term IM therapy[327].

In conclusion these data show that disease persistence is a relevant clinical problem for the ever growing population of CML patients as it has significant financial and social consequences, while also putting patients at risk of disease progression because of both lack of adherence and minimal residual disease persistence.

1.3.7.4.1 Mechanisms of disease persistence and current therapeutic options

The cause of disease persistence in CP CML patients treated with IM has been a subject of intense investigation in the CML research community for the last decade. Soon after the original demonstration of the existence of a highly quiescent CML LSC population, Graham *et al* showed through cell cycle analysis experiments on SPC from CP CML patients that the same population of quiescent LSC was insensitive to induction of apoptosis by IM[328]. The inability to target the LSC was then observed also for DA[329], NL[330] and BOS[331] thus suggesting this is a common feature of all available TKI therapies. These *in vitro* findings have been supported by clinical data from several groups showing that CP CML patients, even after achieving a CMR with TKI (it is noteworthy again that some of the patients analysed had been treated with DA), still harbour phenotypically and functional (both in *in vitro* and *in vivo* assays) *BCR-ABL* positive -both by polymerase chain reaction (PCR) and fluorescence in situ hybridisation (FISH)- LSCs within their stem cell compartment[332,333]. Original mathematical models of the kinetics of the molecular response to IM also suggested that while inhibiting the production of differentiated leukaemic cells, IM did not eradicate the LSC[334]. More recently similar mathematical

modelling of the molecular disease levels and relapse kinetics of patients within IM stopping trials has supported a view where treatment with IM selects a LSC clone with slower growth rate which contributes to disease relapse upon discontinuation of therapy, although to a different rate in each patient[335]. While it is possible that in some patients this clone might not actually lead to overt relapse, speculatively as a result of concomitant factors, such as immunological control, a population of "therapy selected" LSC are still present in all patients. Interestingly experimental data have also supported the idea that persistent disease is correlated to the selective survival of a LSC clone with low *BCR-ABL* expression levels which appears to be less sensitive to IM[336]. Taken together, all the above data support a model where TKI are able to eradicate leukaemia progenitor cells (LPC) and might even produce a selection pressure on LSCs, but they are still unable to completely deplete them.

Having shown that LSCs persist during TKI therapy, understanding the mechanisms causing this phenomenon has been the next logical step. One theoretical possibility is that TKI do not effectively inhibit the BCR-ABL kinase in this selected population of LSCs. However work performed independently from two groups using complementary mouse models and primary CML samples has now shown that TKI are capable of suppressing BCR-ABL TK activity in the most primitive, quiescent LSCs which however still survive while maintaining their functionality as shown by their ability to produce LTC-IC or generate disease in mice. This has therefore proven that CP CML LSCs are not oncogene addicted, or more precisely, that their survival is not dependent on BCR-ABL kinase activity[337,338].

The overall conclusion from this body of work is that the TK activity of the BCR-ABL oncoprotein is not a legitimate therapeutic target to eradicate primitive LSCs which depend on their own intrinsic mechanisms for their survival. Identifying such mechanisms will then provide rational therapeutic targets for developing CML LSC specific drugs, which combined with debulking agents, such as TKI, may lead to disease cure as shown in the model presented earlier in figure 1-10. Recently some of these survival pathways have started to be elucidated and their role as therapeutic targets explored. A detailed presentation of all these newly discovered survival pathways is beyond the scope of this chapter, it is however useful to discuss some general points while presenting some of the most notable ones. A didactic classification of the possible approaches to eradicate LSC divides them into two broad groups. The first group comprises those approaches aiming to reduce CML LSC self-renewal/maintenance capacity mainly by targeting regulators of

their quiescence thus leading to their functional exhaustion. The second group instead aims to identify survival pathways particularly relevant to CML LSC in the specific context of TKI therapy that when targeted might lead directly to LSC apoptosis.

Within the first group, two notable examples have been provided by the TGF-β1/AKT/FOXO axis and the promyelocytic leukaemia (PML) protein, which have both been demonstrated to play a central role in CML LSC maintenance. In the first case, investigators have shown that *BCR-ABL* transduced murine BM cells are not capable of sustaining leukaemia in serial transplantation upon genetic deletion of the *Foxo3a* transcription factor. Moreover using a TGF-β1 R1 kinase inhibitor in combination with IM prolonged survival of CML mice which correlated with downregulation of FOXO3a activity within the LSC[111]. Similarly the PML protein, known to be involved in the development of acute promyelocytic leukaemia, was demonstrated to be overexpressed in CML LSCs and to prevent them entering cell cycle. Murine models lacking *Pml* expression were again shown to be unable to sustain CML development on serial transplantation as their LSCs appeared to exhaust. In support of this model, combined treatment of CML mice with arsenic trioxide, a drug known to target PML for degradation, and standard cytotoxic chemotherapy achieved complete eradication of leukaemia[339].

Within the second type of mechanism (LSC survival pathways), it is worth mentioning the role of the sirtuin 1 (SIRT1) histone deacetylase and autophagy. SIRT1 is highly expressed in CML LSC and inhibits p53 function through deacetylation. The p53 transcription factor plays a central role in inhibiting cancer cell proliferation and inducing apoptosis and is often mutated in cancer cells. SIRT1 inhibition using RNA interference or a small molecule inhibitor, in both primary cells and mouse models, was able to increase p53 transcriptional activity in CML LSCs, thus leading to their more effective eradication when treated with IM[340]. Similarly autophagy is an adaptive process normally activated in eukaryotic cells in response to stress conditions which allows them to adapt to environmental signals. It is based on the breakdown of intracellular materials within lysosomes and, depending on the cell context, can act either as a cell death or as a cell survival mechanism. CML LSCs induce autophagy in response to inhibition of survival signals emanating from BCR-ABL kinase upon TKI treatment to evade cell death. Suppression of autophagy, either using small molecule inhibitors or RNA interference of essential autophagy inducing genes, has been shown to enhance LSC death induced by TKI both in primary CML samples and complementary mouse models[341].

In conclusion this brief and not fully comprehensive overview of the possible survival mechanisms inherent to CML LSC shows that, as might have been expected, not one single mechanism is in control of CML LSC survival and their relative contribution within each patient and between patients ideally needs to be taken into account when devising LSC targeted therapies. This issue coupled with the difficulty of recruiting patients on long-term IM therapy, who have a nearly normal quality of life, into clinical studies probably represent the main reasons for the lack of clinical trials investigating the role of all these specific LSC druggable targets in CML patients with persistent disease. Nevertheless it is worth noting that in some cases clinical testing of these targets is being performed as is the case with the ongoing CHOICES (Chloroquine and imatinib combination to eliminate stem cells) study which our group in Glasgow has devised and is currently conducting in collaboration with several other centres throughout Europe (http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=8492). This is a phase II clinical study looking at the combination of autophagy inhibition using hydroxychloroquine with standard IM therapy in CML patients with persistent minimal residual disease to assess its safety and efficacy in CML LSC eradication. The biggest challenge in the future for the CML community will be to ensure that, as for CHOICES, other clinical studies will be conducted to allow the translation to the bed side of all these exciting discoveries in CML LSC biology.

1.4 Aims

Based on our current knowledge of CML LSC biology and the currently unanswered research questions and clinical problems in the CML field, the overall aim of the work presented in this thesis was to study in further detail the role that GFs play in survival, proliferation and in inducing quiescence/maintenance of CP CML SPC in order to characterise better their exact role in disease persistence. Although some GFs function in CML LSC biology has already been investigated, there is little doubt that our understanding of the role of several other GFs and novel extrinsic regulators of haemopoiesis in CML SPC biology is still lagging behind compared to our knowledge of their role in the normal setting. Moreover the consequences of the presence of the *BCR-ABL* oncogene on CML cell production and response to several GFs is still unknown. Even more, the characterisation at molecular levels of cytokine's mechanisms of action in CML cells is an area requiring further investigation. Finally, an improved understanding of the effects of several GFs on CML cells at biological and molecular level could also have high therapeutic relevance as it might allow identification of novel therapeutic targets to be exploited for the eradication of CP CML LSCs.

As excessive autocrine secretion of some GFs has already been demonstrated as an aberrant feature of CML SPC compared to their normal counterparts, this mechanism was investigated further in an attempt to identify novel CML $CD34^+$ cells survival and proliferation regulators, which are produced autonomously by the CML cells and in a differential way compared to normal cells. While studying in further detail the role of CML autocrine GF production may provide a better understanding of cell autonomous cytokine signals used by CML cells to regulate their survival, the role of truly extrinsic regulators was also investigated, in particular focusing on those whose role in CML is currently unknown, while it has been demonstrated, or is being investigated, in normal haemopoiesis. Following the results of a genome and epigenome-wide analysis of CML and normal stem cells carried out in our laboratory and the recent interest regarding the role of neuromediators in normal haemopoiesis, their putative effects on CML LSC biology was chosen as another topic of investigation. In conclusion the main aims of the work presented in this thesis and the plan of the investigations necessary to achieve them can be summarised in the following points:

1) Characterisation in more detail of the autocrine production of cytokine and cognate receptors in different subsets of normal and leukaemic cells and its modulation by BCR-ABL kinase activity in CML SPC.

2) Characterisation of the functional role of the most interesting autocrine GFs identified from aim 1, by specifically looking at the effects of either inhibiting or stimulating their production/downstream signals at both biological and molecular level.

3) Investigation of the role of several neurotransmitters on survival, proliferation and maintenance of CML LSC using both functional biological and molecular assays.

2 Materials and Methods

2.1 Materials

2.1.1 Small molecule inhibitors

IM was provided as a white powder under a Materials Transfer Agreement (MTA) from Novartis Pharma. It was dissolved in sterile water and stored as a 100mM stock solution at 4°C.

DA, NL, INCB18424 (INC) and TG101209 (TG) were provided in powder form under MTA respectively from Bristol-Myers Squibb (DA), Novartis Pharma (NL and INC) and TargeGen, Inc. (TG). They were all dissolved in anhydrous dimethyl sulfoxide (DMSO) and stored as a 20mM stock concentration at -20°C (DA) and as a 10mM stock solution at -20°C (NL, INC, TG).

TNF-α inhibitor (TNF-α inh) and the TGF-β1 R1 kinase inhibitor LY364947 (LY) were purchased respectively from Merck Chemicals and Sigma-Aldrich, UK, dissolved in DMSO and stored as a 10mM stock concentration at -20°C.

All the inhibitors were made up fresh and diluted to the appropriate concentration with Phosphate Buffered Saline (PBS) prior to use.

2.1.2 Tissue cultures supplies

Abbot Diagnostics, UK	LS1 *BCR-ABL* dual colour FISH probe
Baxter Healthcare, UK	Sterile water
Biolegend, UK	Mouse IgG1, κ isotype control antibody
Becton Dickinson, UK	Luer Lok syringes 19G Syringe needles
BD Biosciences, UK	6-well collagen coated plates Brefeldin A (protein transport inhibitor)
Cell Signaling (New England Biolabs), UK	Human TNF-α
Chugai Pharma, UK	Human G-CSF
Greiner bio one, UK	25 and 75mm^3 tissue culture flasks 6,12,24,48 and 96-well plates FACS tubes Pipette tips
	2-Mercaptoethanol 14.3M KaryoMax Colcemid solution (10μg/mL) Foetal Calf Serum (FCS) L-Glutamine 200mM PBS

Invitrogen, UK	Penicillin-Streptomycin Solution (10,000U/ml) Roswell Park Memorial Institute (RPMI) 1640 media Dulbecco's Modified Eagle Medium (DMEM) Iscove's Modified Dulbecco's Medium (IMDM) Minimum Essential Medium-α (MEM-α) Collagenase IV Collagen coated 6 well plates Non-essential aminoacids solution (NEAA)
Miltenyi Biotec, UK	CliniMACS CD34 reagent CliniMACS PBS/Ethylenediaminetetraacetic acid(EDTA) Buffer CliniMACS tubing set
Nalge Nunc International, Denmark	25 and 75mm^3 non-adherent tissue culture flasks Cryotubes Cryofreezer container
Peprotech, UK	Human TGF-β1 Human FLT-3 ligand (FLT-3L) Human IL-3 Human IL-6 Human stem cell factor (SCF)
R&D Systems, UK	Mouse anti-human TNF-α antibody
Sarstedt, Ltd, UK	5-10-25mL pipettes 15-50mL falcon tubes
Sartorius, Germany	Minisart 0.2μM sterile filters Minisart 0.45μM sterile filters
Scottish National Blood Transfusion Service, UK	20% Human Albumin Solution (ALBA) 4.5% ALBA
Sigma-Aldrich, UK	Bovine serum albumin (BSA) >96% Carbonate-bicarbonate buffer DMSO >99.9% Ethanol >99.5% Histopaque solution (1.077g/mL) Hank's buffered salt solution – calcium and magnesium free (HBSS-CMF) Hydrochloric acid (HCl) 36.5-38% Acetic acid >99.7% PBS tablets Low density lipoprotein (10mg/mL) Magnesium chloride ($MgCl_2$) 1M Methanol 99.8% Pepstatin A protease inhibitor (1mg/mL) Sodium azide 0.1M Trisodium citrate 0.155M Trypan blue 0.4% Trypsin-EDTA 0.25% DL-Norepinephrine hydrochloride Serotonin hydrochloride

	L-Glutamic acid Histamine dihydrochloride Acethylcoline chloride
Stem Cell Technologies, Canada	Bovine pancreatic deoxyribonuclease (DNAse I) 1mg/mL Bovine serum albumin/insulin /transferrin (BIT) serum substitute Hydrocortisone 21-hemisuccinate Methocult H4034 Myelocult H5100 Human GM-CSF Macrophage inflammatory protein-1α (MIP-1α) Leukaemia inhibitory factor (LIF) solution (10μg/mL)
Thermo Fisher Scientific, UK	Sterile cell strainer 70μm mesh
Weber Scientific International, UK	Hawksley Neubauer counting chamber

2.1.3 Flow cytometry reagents

Abcam, UK	Mouse IgG, isotype control antibody Rabbit IgG, isotype control antibody
BD Biosciences, UK	Annexin-V binding buffer (10x) Mouse anti-human IgG Fluorescein isothiocyanate (FITC) isotype control antibody Mouse anti-human IgG Phycoerythrin (PE) isotype control antibody Mouse anti-human IgG allophycocyanin (APC) isotype control antibody FACS flow FACS clean Mouse anti-human-Ki67 FITC antibody Annexin-V FITC/PE/APC Mouse anti-human-CD34 APC antibody Mouse anti-human-CD38 FITC/PE/ Peridinin chlorophyll protein (PerCP-Cy5.5) antibody Viaprobe- 7 aminoactinomycin D (7-AAD) Mouse anti-human-phospho-AKT PE antibody
Biolegend, UK	Mouse anti-human-TNF-α PE antibody
Cell Signaling (New England Biolabs), UK	Rabbit anti-human-phospho-CrKL (p-CrKL) antibody Rabbit anti-human-phospho-STAT5 (p-STAT5) antibody Rabbit anti-human-phospho-NFκB (p-NFκB) antibody Rabbit anti-human-phospho-IκBα (p-IκBα) antibody

Ebiosciences, UK	Mouse anti-human-CD131 PE antibody
Epitomics, USA	Rabbit anti-human-phospho-JAK2 (p-JAK2) antibody
Invitrogen, UK	Fix&Perm cell fixation and permeabilisation kit CellTrace Violet cell proliferation kit CountBright Absolute counting beads CellTrace Carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation kit
Sigma-Aldrich, UK	Goat anti-rabbit IgG FITC conjugate antibody Goat anti-rabbit IgG PE conjugate antibody Formaldehyde solution 36.5% Triton-X-100 1.06g/mL

2.1.4 Molecular biology supplies

Applied Biosystems, USA	High capacity complementary deoxyribonucleic acid (cDNA) reverse transcription kit TaqMan Universal PCR mastermix TaqMan PreAmp mastermix kit Nuclease free water Arcturus Picopure ribonucleic acid (RNA) isolation kit
Bioline, UK	HyperPAGE protein marker
Bio-Rad, USA	Immun-Blot polyvinylidene fluoride (PVDF) membrane Immun-Star WesternC Kit
Cell Signaling (New England Biolabs), UK	Anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody Anti-mouse IgG HRP-linked secondary antibody Rabbit anti-human-β-Tubulin antibody Rabbit anti-human-Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody
Chemicon International USA	Re-blot Plus Strong Antibody Stripping Solution
Epitomics, USA	Rabbit anti-human-Apolipoprotein-E (APO-E) antibody
Invitrogen, UK	NuPage MOPS Sodium dodecyl sulphate (SDS) Running Buffer (20X) NuPage Novex Bis- Tris (hydroxymethyl)aminomethane hydrochloride (Tris) gels NuPage Transfer Buffer (20X) XCell II Blot Module XCell SureLock Mini-Cell

	electrophoresis system NuPage LDS sample buffer (4x) NuPage Reducing agent (10x) CellsDirect One-Step qRT-PCR kit 1xTE Buffer SUPERase-In Tracklt 1KB DNA Ladder 100mM deoxyribonucleotides (dNTP) Set
Fluidigm, USA	48x48 gene Dynamic Array IFC 20x GE Sample loading reagent 2x Assay loading reagent
Pierce ThermoScientific, USA	Bicinchoninic acid (BCA) protein assay kit
Promega Corporation, USA	GoTaq Flexi DNA Polymerase (5u/µL), including $MgCl_2$, and loading buffer
Qiagen, Crawley, UK	RNeasy mini kit RNeasy micro kit
Roche Diagnostics, Germany	cOmplete, EDTA-free Protease inhibitor cocktail tablets PhosSTOP, Phosphatase inhibitor cocktail tablets
Sigma-Aldrich, UK	EDTA Ethidium bromide solution 10mg/mL Formaldehyde solution 36.5% NP-40 (1.06g/mL) Radioimmunoprecipitation assay (RIPA) buffer Sodium chloride (NaCl) >99% Tris base >99% Sodium deoxycholate >97% Tween-20 (1.095g/mL)

2.1.5 Custom designed PCR primer sequences and TaqMan Gene expression assays

See Appendix I

2.2 Preparation of media and solutions

2.2.1 Tissue culture media

2.2.1.1 RPMI^{++}

RPMI 1640	500mL
FCS	50mL
L-glutamine	5mL
Penicillin/Streptomycin solution	5mL

2.2.1.2 MEM-α^{++} for maintenance of stromal cell line OP-9

MEM-α	500mL
FCS	100mL
L-glutamine	5mL
Penicillin/Streptomycin solution	5mL
NEAA	5mL
2-Mercaptoethanol	500µL

2.2.1.3 Serum free medium (SFM)

BIT	25mL
2-Mercaptoethanol	250µL
L-glutamine	1.25mL
Penicillin/Streptomycin solution	1.25mL
IMDM	97.25mL
Low density lipoprotein	500µL

Made up in a Vacubottle and filter sterilised

2.2.1.4 SFM supplemented with high 5 GF cocktail (SFM+HiGF)

SFM	50mL
IL-3 (50µg/mL)	20µL
IL-6 (50µg/mL)	20µL
G-CSF (20µg/mL)	50µL
FLT-3L (50µg/mL)	100µL
SCF (50µg/mL)	100µL

Filter sterilised through 0.22µM syringe filter

2.2.1.5 SFM supplemented with physiological GFs (SFM+PGF)

SFM	50mL
SCF (0.5µg/mL)	20µL
G-CSF (2µg/mL)	25µL
IL-6 (5µg/mL)	10µL
GM-CSF (0.1µg/mL)	100µL
LIF (0.1µg/mL)	25µL
MIP-1α (0.1µg/mL)	100µL

Filter sterilised through 0.22µM filter

2.2.1.6 Myelocult

Myelocult H5100	100mL
Hydrocortisone hemisuccinate ($1x10^{-4}$M)	1mL

2.2.2 Tissue culture solutions

2.2.2.1 PBS/2% FCS

PBS	490mL
FCS	10mL

2.2.2.2 PBS/20% FCS

PBS	80mL
FCS	20mL

2.2.2.3 DAMP solution for thawing cryopreserved $CD34^+$ cells or MNC aliquots from -150°C

DNAse II solution	1 vial
$MgCl_2$	625µL
Trisodium citrate	26.5mL
20% ALBA	12.5mL
PBS	to 250mL

2.2.2.4 20% DMSO/4.5% ALBA

DMSO	20mL
4.5% ALBA	80mL

2.2.2.5 IMDM/ 2% FCS

IMDM	98mL
FCS	2mL

2.2.3 Flow cytometry solutions

2.2.3.1 PBS/0.4% formaldehyde

PBS	48mL
10% formaldehyde	2mL

2.2.3.2 PBS/0.2% Triton-X-100

PBS	50mL
Triton-X-100	100µL

2.2.3.3 Annexin-V binding buffer

Annexin-V binding buffer (10X)	1mL
Distilled water	9mL

2.2.3.4 Fix&perm wash – PBS/1% BSA

BSA	10g
PBS	to 1L

2.2.4 Molecular biology solutions

2.2.4.1 Lysis buffer for protein lysates (RIPA)

Distilled water	7.75mL
1.5M NaCl	1mL
1M Tris-HCl	0.5mL
150mM EDTA	333µL
NP-40	50µL
10% Sodium deoxycholate	250µL
Protease inhibitor (10X)	100µL
Phosphatase inhibitor (10X)	100µL

2.2.4.2 Running buffer

NuPage MOPS SDS Running buffer (20X)	30mL
Distilled water	to 600mL

2.2.4.3 Transfer buffer

NuPage transfer buffer (20X)	30mL
Distilled water	510mL
Methanol	60mL

2.2.4.4 Tris-buffered saline (TBS) 10X

NaCl	876.6g
Tris base	121.1g
Distilled water	to 10L

Adjusted to pH 8.0

2.2.4.5 1X TBS-T buffer/ wash buffer

TBS 10X	1L
Tween 20	10mL
Distilled water	to 10L

2.2.4.6 Blocking buffer

1X TBS-T	100mL
BSA	5g
dry, skimmed milk	5g

Either BSA or dry/skimmed milk were used depending on antibody

2.2.4.7 10XTBE buffer

Boric acid	55g
EDTA (0.5M, pH 8.0)	40ml
Tris base	108g
Distilled water	1L

2.2.4.8 2X reverse transcription (RT) Mastermix

10X RT Buffer	2µL
25X dNTP Mix	0.8µL
10X RT Random Primers	2µL
Reverse Transcriptase 50u/µL	1µL
RNase Inhibitor	1µL
Nuclease free water	3.2µL

2.2.4.9 Reverse Transcription (RT)-PCR mix

5X Green GoTaq Flexi Buffer	5µL
10mM dNTP Set	0.5µL
5' and 3' primers (25µM)	0.2µL
GoTaq DNA polymerase	0.1µL
$MgCl_2$ Solution (25mM)	3µL
cDNA+ Nuclease free water	16µL

2.2.4.10 qRT-PCR Mastermix (TaqMan)

2X TaqMan Universal PCR Mastermix	5µL
20X TaqMan gene expression assay	0.5µL
cDNA+ Nuclease free water	4.5µL

2.2.4.11 Specific target amplification Mastermix

2X TaqMan Preamplification Mastermix	12.5µL
0.2X pooled gene expression assay mix	6.25µL
cDNA+ Nuclease free water	6.25µL

2.2.4.12 Single cell RNA extraction/cDNA/preamplification Mastermix

2X cell reaction mix	5µL
0.2X pooled gene expression assay mix	2.5µL
SUPERase-In	0.1µL
Superscript III RT/Platinum TaqMix	0.2µL
1xTE buffer	1.3µL

2.2.4.13 qRT-PCR assay mix (Fluidigm)

2x assay loading reagent	2.5µL
20X TaqMan Gene expression assay	2.5µL

2.2.4.14 qRT-PCR sample mix (Fluidigm)

2X TaqMan Universal PCR Mastermix	2.5µL
20x GE sample loading reagent	0.25µL
cDNA	2.25µL

2.3 Methods

2.3.1 Cell culture and cellular techniques

2.3.1.1 Culture of cell lines

The BC CML cell lines K562 and KCL22 (BCR-ABL positive) and the AML cell line HL60 (BCR-ABL negative) available in house were grown in suspension culture in $RPMI^{++}$ medium. All cell lines were maintained at 37°C with 5% CO_2 in $25cm^3$ tissue culture flasks. Cells in suspension were counted and passaged every two days with warm fresh medium, to maintain a density of between $1x10^5$-$1x10^6$ cells/mL. Prior to each experiment cells were washed, resuspended in fresh medium and seeded at a density of $2x10^5$/mL for each treatment arm. Cells were harvested at different time points for protein and gene expression analysis and functional assays as dictated by experimental design.

The murine green fluorescent protein (GFP) tagged OP-9 stromal cells available in house were cultured in MEM-α^{++} medium and passaged after reaching confluency. For the coculture experiments, $5x10^4$ OP-9 cells per well were seeded in 6 well collagen-coated plates with fresh medium. Fresh medium was replenished after 3 days and prior to plating primary haemopoietic cells.

2.3.1.2 Primary CML and normal $CD34^+$ cells collection and enrichment

All samples were collected with the approval from the Local Research and Ethics Committee and with written informed patient consent. Samples were obtained from patients at diagnosis of CP CML prior to any drug treatment, patients with normal BM stem cells undergoing autologous stem cell collection and normal healthy volunteers.

Either 6mL of histopaque solution was added to a 15mL falcon tube, or 20mL of histopaque solution was added to a 50mL falcon tube (depending on the volume of blood sample) and brought to room temperature. The whole blood sample was first diluted (1:2) with PBS, carefully layered drop-wise onto the histopaque solution, until it reached the top of the centrifuge tube and centrifuged at 400g for 30 minutes at room temperature. Following centrifugation, the opaque interface containing the MNC was carefully aspirated with a 3mL sterile pastette. The interface was then transferred into a sterile centrifuge tube with a pastette and washed twice with sterile PBS (centrifuge at 300g for 5 minutes). The resultant MNC were either cryopreserved or further enriched for $CD34^+$ cells.

$CD34^+$ enrichment was achieved using the CliniMACS system (Miltenyi Biotec, Bisley, UK). MNC were incubated with a specific anti-CD34 monoclonal antibody (Miltenyi Biotec) to which super-paramagnetic MACS beads (~50nM in diameter) had been conjugated. The cell sample was then passed through a high-gradient magnetic separation column, where the target $CD34^+$ cells were retained in the column and the unlabelled $CD34^-$ cells flushed through and discarded. The bound $CD34^+$ cells were then eluted after removal from the magnetic field, collected and an aliquot was removed for flow cytometry assessment of CD34 purity, which confirmed that all samples were >95% $CD34^+$ post-selection. All samples were stored at the indicated concentrations (see Section 2.3.1.3) in cryotubes at -185°C, until required for use. Finally all CP CML samples were determined to be *BCR-ABL*$^+$ by PCR. The preparation of the MNC samples and enrichment for $CD34^+$ cells were kindly performed by Dr Alan Hair.

2.3.1.3 Cryopreservation of cells

Cryopreservation in liquid nitrogen at -185°C of both cell lines and fresh primary cells was used for long term storage. Between $4x10^6$-$2x10^7$ $CD34^+$ selected cells or $1x10^8$ unselected MNC were suspended in 1 to 2mL of an equal volume solution of 20% DMSO in 4.5% ALBA to give a final concentration of 10% DMSO and aliquoted in cryotubes. Likewise, $5x10^6$-$1x10^7$ cell lines in exponential growth were suspended in 1 to 2mL of a solution of 10% DMSO in FCS and aliquoted in cryotubes. The cryotubes were transferred to a cryofreezer container and first cooled at a controlled rate to -80°C to before being transferred to -185°C freezer for long-term storage.

2.3.1.4 Recovery of frozen cells

Primary $CD34^+$ cells and MNC were removed from -150°C and immediately thawed at 37°C in a water bath until the ice crystals had disappeared. Using a pastette, the cells were added to a 15ml sterile tube and recovered by slowly adding 10ml of thawing solution (DAMP) drop-wise over a 20 minute period. This step was performed at room temperature to enhance the activity of the DNAse II, with constant agitation to prevent clumping of the cells. The cells were centrifuged at 300 g for 10 minutes, the supernatant was poured away and the pellet loosened by flicking the tube. The pellet was then washed twice in DAMP and centrifuged, then resuspended in SFM+HiGF or SFM+PGF (depending on the experiments) for counting and cell viability.

Cell lines were thawed in a 37°C water bath and recovered slowly as above but in RPMI^{++} and MEM-α (depending on the cell lines, see Section 2.3.1.1). The cells were then washed twice more and resuspended in 10mL of appropriate medium (see Section 2.3.1.1).

2.3.1.5 Primary CD34$^+$ cells sorting

Cell sorting was kindly performed by Miss Jennifer Cassels. Briefly, fresh or recovered MNC or CD34$^+$ cells were washed, resuspended in PBS/2% FCS and stained with anti-human anti-CD34 APC and anti-CD38 FITC or PECy7 antibodies (depending on the experiments) for 15 minutes in the dark. Small cell aliquots ($2x10^4$) were used for relevant isotype controls and single colour controls to define positive and negative staining, respectively for each marker and to set compensation. Stained cells were washed and filtered through a 0.22μM filter prior to sorting with a BD FACS Aria with Diva software into two populations: CD34$^+$ CD38$^-$ and CD34$^+$ CD38$^+$. The CD34$^+$ CD38$^-$ fraction approximates the most primitive quiescent stem cell pool (less than 5% total CD34+ cells)[342] (figure 2-1) and for all CP CML samples their Ph$^+$ status was confirmed by dual-colour FISH (D-FISH).

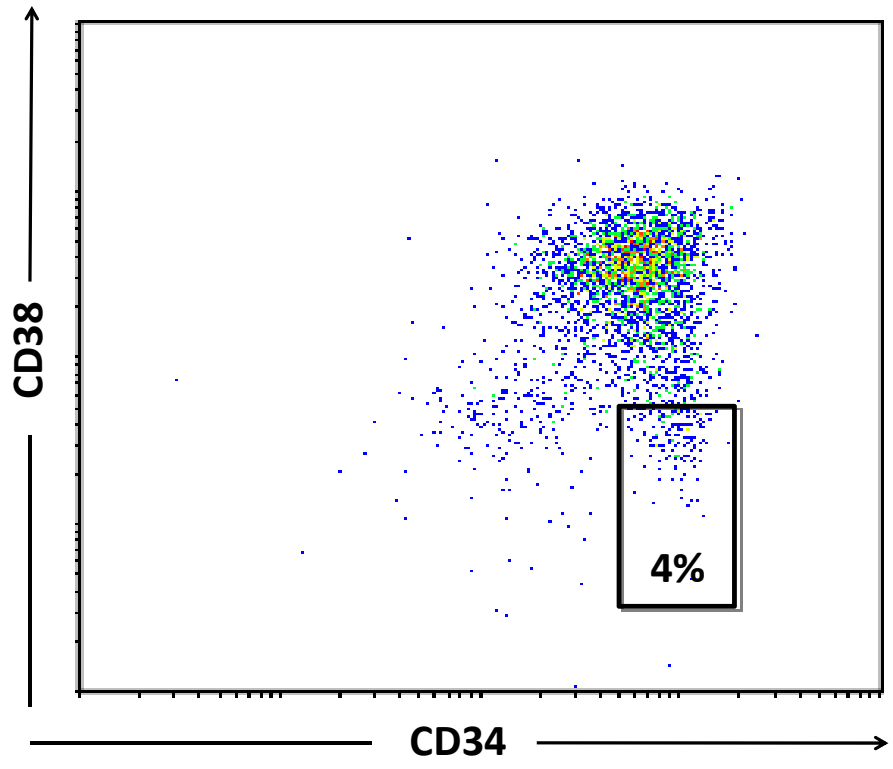


Figure 2-1 Representative plot of the gating strategy used to sort CD34$^+$ CD38$^-$ cells from bulk CD34$^+$ cells
A strict gate based on background matched isotype antibody staining was employed yielding usually between 2 and 5% CD34$^+$ CD38$^-$ cells out of the total CD34$^+$ cells

2.3.1.6 Culture of primary cells

$CD34^+$ cells or sorted subpopulations were maintained in suspension culture in SFM+PGF or SFM+HiGF depending on experimental setup at 37°C with 5% CO_2 in $25cm^3$ tissue culture hydrophobic flasks overnight prior to set up experimental conditions. Prior to each experiment cells were washed, resuspended in fresh medium and seeded at a density of 1-$2x10^5$/mL for each treatment arm. Cells were harvested at different time points for protein and gene expression analysis and functional assays as dictated by experimental design.

In the coculture experiments $5x10^3$ – $5x10^4$ CML $CD34^+$ $CD38^-$ cells for each treatment arm were seeded in each well on top of confluent GFP tagged OP-9 stromal cells and the OP-9 media was replaced with 2mL of Myelocult H5100 supplemented with hydrocortisone sodium succinate at a final concentration of 10^{-6}M. The coculture was maintained for 3 days at 37°C and 5% CO_2 by which point the medium was replaced by 1mL collagenase IV/Myelocult (1mg/mL) solution and incubated for 20 minutes at 37°C. Subsequently, cells were incubated for 15 minutes at 37°C with 1mL of trypsin followed by harvesting using cell scrapers and 1mL FCS to neutralise trypsin. Harvested cells were used for protein and gene expression analysis and functional assays as per experimental design.

2.3.1.7 Cell counting and cell viability assessment

Cell counts and assessment of viability were performed using a counting chamber. Cells were counted with trypan blue exclusion. Trypan blue dye was first diluted 1:10 with PBS and 10μL was added to 10μL of cell suspension to give a 1:2 dilution of cells. Approximately 10μL of the mixture was transferred to a haemocytometer and a minimum of 100 viable cells were counted. Dead cells have damaged membranes and therefore absorb the trypan blue dye, appearing dark blue under the light microscope, whereas live cells with an intact membrane do not absorb the dye. Hence, the unstained cells were counted and the remaining stained cells were deemed non-viable.

2.3.1.8 Colony forming cell (CFC) assay

The CFC assay is an *in vitro* assay specifically developed to quantify the haemopoietic progenitors present within a cell sample by enumerating the number of colonies produced following culture in a semi-solid media in the presence of adequate cytokine support. It can also help to characterise the type of progenitors present in the sample based on the

morphology of the colonies produced. It is based on the known ability of haemopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation[343].

Briefly, following treatment for 3 or 6 days $3x10^5$ cells from each arm were washed and resuspended in 300µL of fresh medium and then added to 3mL of Methocult H4034. This suspension was thoroughly mixed and then 1.3mL was added to a 35mm tissue culture dish in duplicate. An extra 35mm tissue culture dish containing sterile water was added to prevent culture drying. The dishes were incubated for 12-14 days at 37°C, 5% CO_2 prior to counting the number of viable colonies.

The capacity of an individual haemopoietic progenitor cell colony to replate in a CFC assay has been used as an indicator of the self-renewal potential of that progenitor[344,345]. Therefore CFC replating assays were performed to assess drug treatment effects on self-renewal capacity of haemopoietic progenitors.

In replating experiments 50 single colonies from each arm were harvested from Methocult H4034 between day 10-12 of primary culture, resuspended in 10µL of fresh medium and plated in 100µL of Methocult H4034 within each well of a 96-well plate with thorough mixing. The plates were then incubated for further 12-14 days at 37°C, 5% CO_2 prior to counting the number of viable colonies.

2.3.1.9 D-FISH

CML $CD34^+$ and $CD34^+$ $CD38^-$ cells were assessed for the presence of *BCR-ABL* by D-FISH prior to culture. $1x10^5$ cells were washed in PBS/2% FCS and then resuspended in 30µL pre-warmed (37°C) hypotonic solution (0.075M potassium chloride) and spotted to a well of multi-spot microscope slides previously coated with poly-L-lysine and air-dried overnight. Samples were incubated at room temperature for at least 20 minutes. 10µL of freshly made fixative (methanol:acetic acid [3:1]) were subsequently added to the well containing cells and allowed to dry out. This procedure was repeated further 3 times. The prepared slides were wrapped in parafilm and stored at -20°C until D-FISH was performed with the LS1 *BCR-ABL* Dual Colour, Dual Fusion translocation probe according to the manufacturer's instructions. Interphase nuclei were evaluated using a Leica fluorescence microscope with a triple band pass filter for DAPI, Spectrum Orange and Spectrum Green. All D-FISH slides were kindly prepared and scored by Mrs Elaine Allan.

2.3.2 Flow cytometry

Flow cytometry is a technique able to characterize and sort samples at single cell level using fluorochrome-labelled antibodies and dyes. Single cells in a stream of fluid are presented to a laser which excites the fluorescent dyes. The fluorochromes then emit light at different wavelengths (colours) allowing simultaneous measurement of several different properties of a single cell. Finally it also measures size and granularity of single cells by their forward-angle and side-angle light scatter, respectively (FSC and SSC)[346].

2.3.2.1 Detection and quantification of protein expression

To detect and quantify levels of expression of protein by flow cytometry, between $5x10^4$ and 1×10^5 primary cells were harvested and washed in PBS/2%FCS. For surface protein staining cells were resuspended in 100µL PBS/2% FCS with appropriate antibody solution or appropriate isotype antibody as negative control for 15 minutes in the dark. After incubation, the cells were washed in PBS/2% FCS and either analysed by flow cytometry immediately or further processed depending on the experimental setup. For intracellular protein staining cells were washed and resuspended in 100µL of fixing reagent (reagent A of Fix&Perm cell fixation and permeabilisation kit) for 15 minutes. The cells were then washed in PBS/1%BSA and the supernatant completely removed. The cell pellet was subsequently resuspended in 25µL of permeabilising reagent (reagent B Fix&Perm cell fixation and permeabilisation kit) containing the relevant antibody for 60 minutes. Excess antibody was then washed prior to flow cytometry analysis. When an unconjugated primary antibody was used, the cells were washed and resuspended in 100µL of PBS/1%BSA with either 2µL of the secondary anti-rabbit IgG FITC conjugate (1:50 dilution), or 10µL of the secondary anti-rabbit IgG PE conjugate (1:10 dilution) depending on the requirement for multi-parametric flow cytometry analysis, at room temperature in the dark for 30 minutes. Quantification of the protein of interest within each sample was calculated in relative terms, using the ratio between the geometric mean fluorescence intensity (MFI) of the antibody labelled live cells and the geometric MFI of the isotype labelled live. When measuring changes in levels of protein within each sample following treatment, the MFI ratio (calculated as explained above) for each treatment arm was expressed as a percentage of the value of the untreated (UT) control (100%).

2.3.2.2 High resolution cell cycle analysis

Flow cytometry can effectively be used for assessment of the cell cycle status of cells within each sample and following treatment. To this end a staining combining detection of the intranuclear antigen Ki67 together with the DNA binding dye 7AAD was used. Ki67 is an antigen present in the nuclei of cells which are in the active phases of cell cycle - G1, S/G2 and M. It is not expressed in G0 cells and therefore is present only in proliferating cells[347] so is commonly used as a marker for cellular proliferation. 7AAD allows measurement of DNA content within each cell. By combining Ki67 with 7AAD, it is possible to distinguish cells in the different phases of cycle[348]. G0 cells will have low (diploid) DNA content and do not express Ki67. Cells in G1 phase have the same DNA content but begin to express Ki67. Following replication of the DNA during the S phase, the DNA content within each cell effectively doubles and Ki67 expression also increases as cells progress from S phase to G2 and then mitosis (figure 2-2).

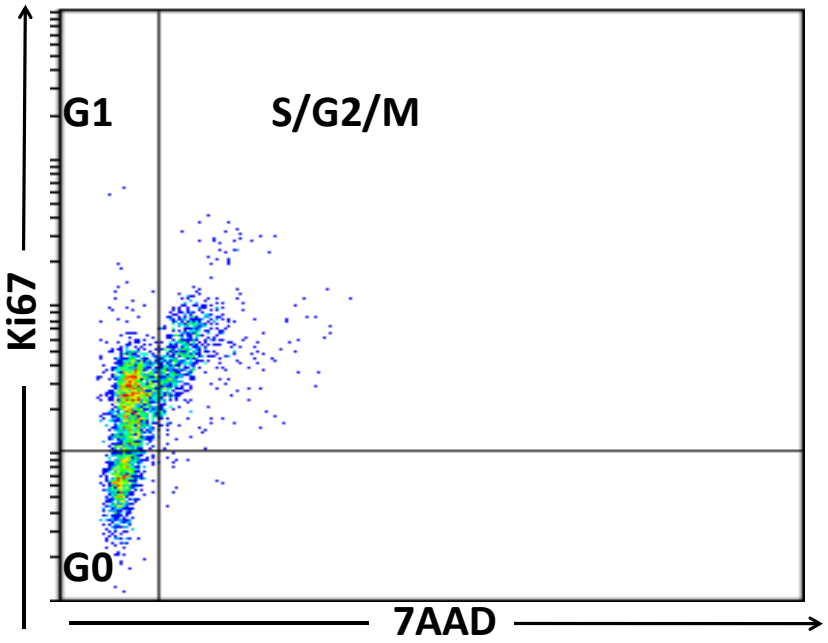


Figure 2-2 Representative plot of high resolution cell cycle staining by flow cytometry G0 cells have undetectable expression of Ki67 and low DNA content (lower left). G1 cells have low DNA content but express Ki67 (upper left) and S/G2/M cells have double DNA content and high level of expression of Ki67 (upper right)

Practically $2x10^5$ cells were washed in PBS/2%FCS and then resuspended in 500µL of PBS/0.4% formaldehyde for fixing for 30 minutes on ice. Following this, the cells were permeabilised in 500µL of PBS/0.2% Triton-X-100 overnight at 4°C. The following morning, the cells were washed once in PBS/2%FCS and stained with either 20µL of Ki67 FITC labelled antibody or 20µL of FITC isotype control for 40 minutes at room

temperature in the dark. Following one further wash, the cells were resuspended in 1mL PBS with 5µL of 7AAD (1µg/mL) for 6 hours. Before flow cytometry analysis, the cells were washed once more in PBS/2%FCS. Cells stained with levels of 7AAD below G0 levels were gated out from analysis as they represented dead cells and debris. Within the live cell population, the relative percentages of cells at each stage of the cell cycle were then measured by combining the Ki67 and 7AAD staining.

2.3.2.3 CFSE staining

The CFSE fluorescein-based dye staining of cells is a technique used to track cell division by flow cytometry. It was first described by Lyons and Parish in 1994 to determine divisions of lymphocytes[349]. CFSE is a lipophilic molecule which upon entering the cells is converted to a reactive dye by non-specific intracellular esterases. In the cell, it binds irreversibly to the free amines of cytoplasmic proteins and therefore allows stable cell staining. However upon division, the dye is equally divided between the two daughter cells determining a serial dilution of the dye with each division. As a result the fluorescence intensity of cells is halved with every cell division allowing a measure of the number of divisions a sample has undergone in culture, i.e. its proliferation (figure 2-3).

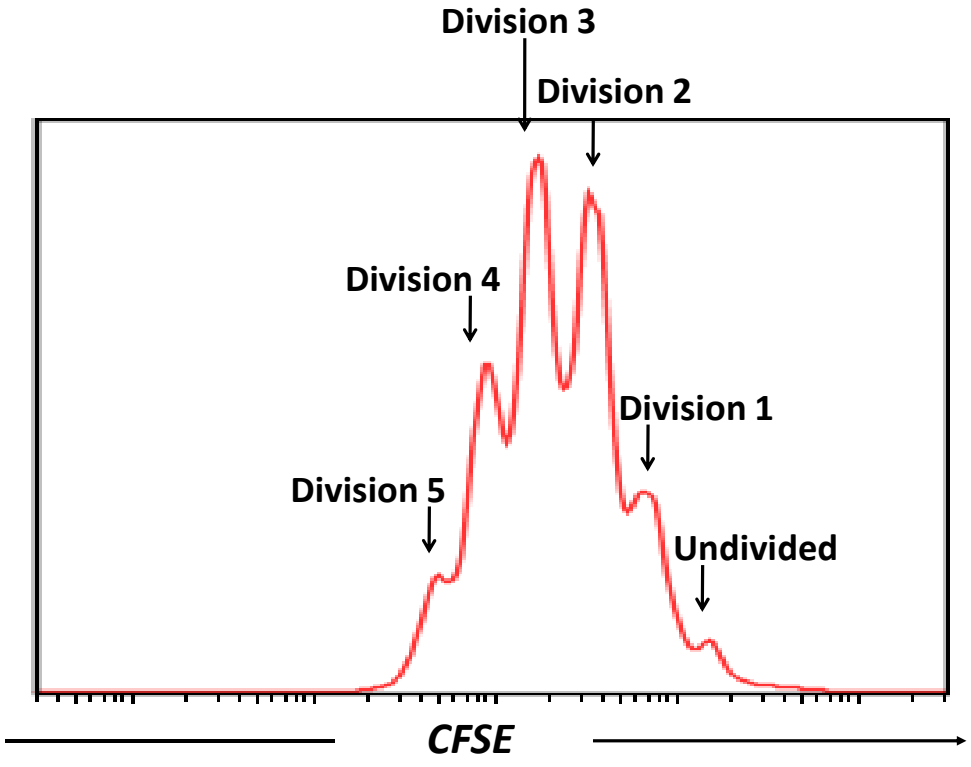


Figure 2-3 CFSE plot showing halving of CFSE fluorescence intensity with each division

Practically, following recovery and resuspension in 5mL PBS/2%FCS, $CD34^+$ cells were stained with 10μL of a 500μM solution of CFSE dye to give a final concentration of 1μM for 10 minutes at 37°C in a water bath, while a small aliquot of cells was kept unstained for control purposes. Following this, CFSE was quenched by adding 10x volume of ice cold PBS/20% FCS and then washed once more time in fresh PBS/2% FCS. The cells were cultured overnight and the following day the total number of viable cells was recorded by flow cytometry using a 200μL aliquot of the cell suspension to which 20μL of a known concentration of counting beads for flow cytometry (CountBright Absolute counting beads) had been added. This also allowed confirmation of uniform CFSE staining and recording of the position of the undivided ($CFSE^{max}$) cell population prior to culture in different treatment conditions. Moreover to confirm the position of undivided cells following treatment, a control well was set up to which 100ng/mL of Colcemid was added which effectively blocked cell proliferation.

As well as allowing detection of the number of divisions a sample has undergone in culture, CFSE stain can also be used to track the fate of all the cells in culture by measuring the percentage recovery of viable input cells within each division following treatment. To this purpose both the number of viable cells seeded initially in each culture and their number following different treatment conditions is recorded by bead counting as explained above. The level of CFSE fluorescence is used to identify cells that have undergone 0, 1, 2, 3 etc. divisions and by gating on each division peak it is possible to measure the percentage of cells within each division. Following this, the absolute number of input cells which have undergone 0, 1, 2, 3 etc. divisions can be calculated using the formula shown in table 2-1:

Absolute number of input cells remained undivided	**=**	**Absolute number of viable cells following treatment*percentage of cells in undivided CFSE gate/1**
Absolute number of input cells undergone 1 division	**=**	**Absolute number of viable cells following treatment*percentage of cells in division 1 CFSE gate/2**
Absolute number of input cells undergone 2 divisions	**=**	**Absolute number of viable cells following treatment*percentage of cells in division 2 CFSE gate/4**
Absolute number of input cells undergone 3 divisions	**=**	**Absolute number of viable cells following treatment*percentage of cells in division 3 CFSE gate/8**
Absolute number of input cells undergone 4 divisions	**=**	**Absolute number of viable cells following treatment*percentage of cells in division 4 CFSE gate/16**

Table 2-1 Formula for calculating absolute number of input cells recovered within each division using CFSE staining

Finally the percentage of starting cells recovered within each division can be measured by dividing the absolute number of input cells undergone 0, 1, 2, 3 etc. divisions by the total

number of input cells and expressing this value as a percentage. This analysis allows a comparison of the percentage of input cells recovered within each division following different treatment conditions and is mainly used to detect the effects of different treatments on the undivided, quiescent $CFSE^{max}$ population which has been shown to be enriched for LSCs[192].

Of note in some experiments to allow multi-parametric flow cytometry analysis, a CellTrace Violet stain was used to track cell divisions. This is a stain with similar properties to the CFSE stain but has different emission spectrum thus allowing the combination of cell proliferation analysis with other assays requiring the use of a fluorochrome with the same emission spectrum of standard CSFE.

2.3.2.4 Assessment of apoptosis and viability

Apoptosis induction was measured by flow cytometry using annexin-V staining. During early phases of apoptosis, the phospholipid phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane and exposed to the external cellular environment. Annexin-V, a phospholipid-binding protein, can be used to identify cells undergoing apoptosis when conjugated with a fluorochrome detectable by flow cytometry. Annexin-V staining was used in association with the DNA binding dye 7AAD which is only able to stain dead or late apoptotic cells because their membranes are damaged to such an extent that they cannot exclude the 7AAD dye. As a result it was possible to differentiate live cells (annexin-V and 7AAD negative), cells undergoing early apoptosis (annexin-V positive, 7AAD negative), cells in the late stages of apoptosis (annexin-V and 7AAD positive) and dead cells (annexin-V negative, 7AAD positive) (figure 2-4).

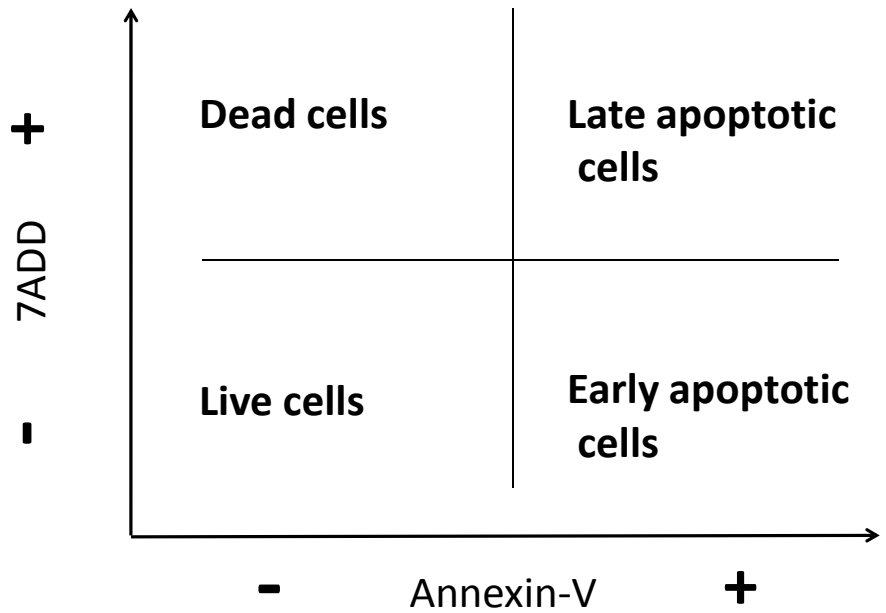


Figure 2-4 Schematic representation of apoptosis and viability assessment by annexin-V and 7AAD staining

Practically, following a wash in PBS/2%FCS 0.5-1x10^5 cells were resuspended in 100µL of annexin-V buffer containing 5µL annexin-V conjugated to relevant fluorochrome and 5µL 7AAD and incubated at room temperature for 15 minutes in the dark. Following incubation, further 200µL of annexin-V buffer was added to the cell suspension and analysis was immediately performed by flow cytometry.

2.3.3 Western blotting

Western blotting measures protein expression in cells following their lysis and fractionation of the protein component. The proteins are first separated according to their size by gel electrophoresis. Thereafter they are transferred or 'blotted' onto a membrane, almost always of nitrocellulose or PVDF, by placing the gel next to the membrane and by applying an electrical current which allows the proteins in the gel to move to the membrane where they adhere. The membrane will therefore contain all the proteins expressed within the cell sample tested. Following incubation with a specific primary antibody against the protein of interest, the membrane is incubated with a secondary antibody coupled to an easily detectable enzyme such as HRP on addition of a substrate. This allows the detection and quantification of a specific protein within each sample tested.

2.3.3.1 Protein lysate preparation

The RIPA lysis buffer was prepared immediately prior to use. Cells from different treatment arms were harvested and washed twice in ice cold PBS at 4°C. The supernatant

was removed carefully with a graduated pipette and the fresh lysis buffer was added to the cells (50µL per $5x10^5$ cells), mixed by pipetting up and down and incubated for 20 minutes on ice. Following this the cell suspension was spun at maximum speed for 10 minutes at 4°C to pellet DNA and debris. The supernatant which contains the protein was then carefully aspirated and either immediately used or stored at -80°C until use.

2.3.3.2 Protein quantification

The BCA method was used for colorimetric detection and quantification of total protein according to the manufacturer's instructions. This assay is based on the reduction of cuprous cation from Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the so called biuret reaction) and the following colorimetric detection of the purple reaction product formed by the chelation of one cuprous cation (Cu^{1+}) with two molecules of BCA^{350}. This water soluble complex exhibits a strong absorbance at 562nm that is nearly linear over a broad range of protein concentrations (20-2,000µg/mL). Protein concentrations are determined with reference to standards of known concentrations of BSA.

The BSA standards were prepared by dissolving a measured amount in 0.9% saline to give a stock concentration of 2,000µg/mL. The following serial dilutions of the stock were prepared to produce a concentration gradient for the controls: 1,500; 1,000; 750; 500; 250; 125; 50; 25; 5 and 0 (blank) µg/mL. 25µL of each serial dilution of the standards was pipetted in duplicate onto the well of a 96-well plate. 5µL of the protein lysate from each sample was added to 20µL of PBS (1:5 dilution) and also pipetted in duplicate onto the well of a 96-well plate. A BCA working solution was prepared by mixing solutions A and B of the BCA protein assay kit in a 50:1 ratio and 200µL of the working solution was added to each well. The 96-well plate was then incubated at 37°C for 30 minutes and the plate was read using an enzyme-linked immunosorbent assay (ELISA) plate reader for absorbance at 562nm. Protein concentrations were calculated with reference to BSA standards so that equal amounts of protein could be loaded for Western blot analysis.

2.3.3.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique used to separate protein in a highly cross-linked gel based on their size and following their denaturation. The anionic detergent SDS binds to the protein molecules in a fixed mass ratio of 1.4:1 and causes their denaturation. This confers an equal negative charge density to each polypeptide and as a result, migration is determined

only by the protein molecular weight. It is usual to associate this procedure with the reduction of disulphide bridges in proteins which is necessary for optimal separation by size. This is normally achieved using a reducing agent such as 2-Mercaptoethanol. Following application of an electric current each negatively charged protein molecule migrates towards the positive electrode. The distance of migration depends on the size of the polypeptides with smaller polypeptides travelling more rapidly through the gel thus allowing protein separation according to their size.

Practically protein lysate was mixed with NuPage LDS 4x sample buffer in a 1.5mL eppendorf and then heated to 70°C for 10 minutes. The samples were then loaded into a NuPage Novex Bis-Tris 4-12% gradient gel. 10µL of HyperPage protein marker was also loaded for protein size assessment. The gel was run using 1X NuPage MOPS SDS Running Buffer in an Invitrogen XCell SureLock Mini-Cell electrophoresis system at 80V for 15 minutes and then 120V till adequate protein separation was achieved.

2.3.3.4 Transfer to PVDF membrane

Transferring or blotting proteins onto a membrane is based on the same principle of gel electrophoresis, i.e. charged proteins can be transferred in an electrical field from the gel onto a robust support such as a PVDF membrane.

Briefly transfer was performed in wet conditions using the Invitrogen XCell II Blot Module at 30V for 60 minutes. The PVDF membrane was first soaked in methanol shortly for its activation, and then in transfer buffer for several minutes. Sponges and filter papers were also soaked in transfer buffer before assembling the transfer sandwich as shown in figure 2-5

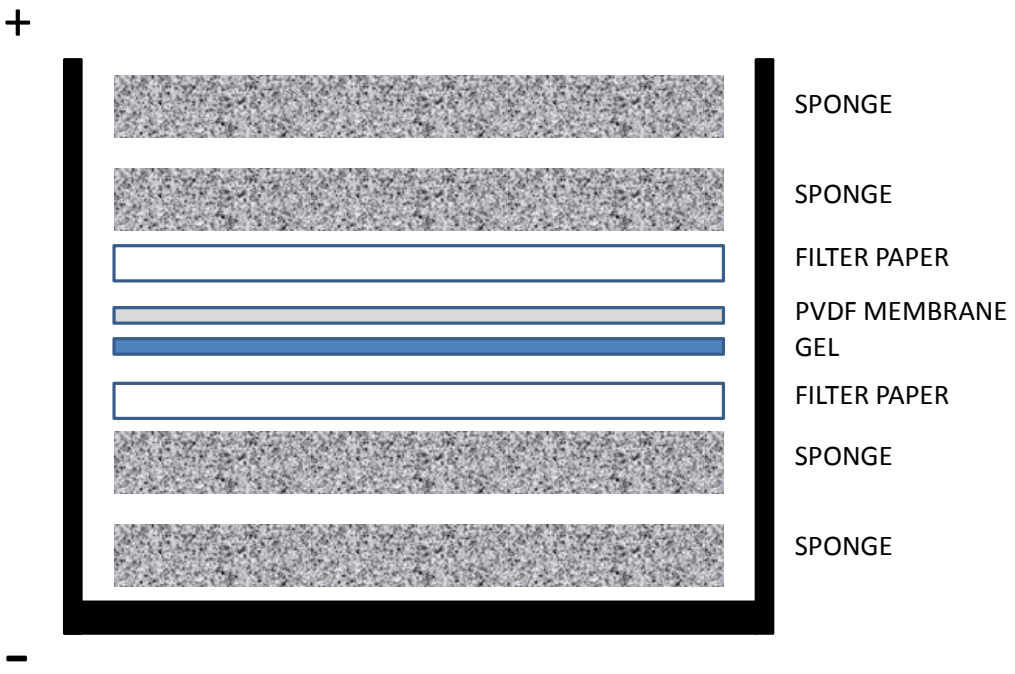


Figure 2-5 Schematic representation of the transfer assembly

10% to 20% methanol was added to transfer buffer both to reduce gel swelling thus increasing protein resolution during transfer and to remove SDS from proteins thus increasing their binding to the membrane.

2.3.3.5 Antibody labelling

Following transfer, the PVDF membrane was briefly washed in TBS-T and then incubated in blocking buffer, with gentle agitation at room temperature for 1 hour. Thereafter the blocking buffer was discarded and the membrane incubated with primary antibody overnight at 4°C with constant rotation. The membrane was then washed the following morning for 4 times (15 minutes per wash) in TBS-T to remove excess primary antibody prior to incubation with appropriate HRP-conjugated secondary antibody (1:3,000 dilution) for 1-2 hours at room temperature with constant rotation. Following 4 more washes (15 minutes per wash) in TBS-T to remove excess secondary antibody, the blot was incubated with the Immun-Star WesternC Kit as per manufacturer's protocol. Immun-Star ECL reagents (luminol/enhancer and peroxide solutions) were mixed in equal amounts before use and incubated with the membrane for few minutes. HRP bound to the secondary antibody oxidises luminol in the presence of hydrogen peroxide. Upon returning to its initial state oxidized luminol emits light. The enhancer in the mixture serves the purpose of

increasing signal intensity and length. Finally digital pictures of protein bands were taken by the Molecular Imager ChemiDoc XRS machine.

2.3.3.6 Stripping and reblotting

Stripping allows sequential incubation of a membrane with different antibodies by removing antibodies bound into the membrane without removing the actual protein. This was performed by incubating the PVDF membrane with the Re-Blot Plus Strong stripping solution diluted 1:10 with distilled water. Following two brief washes (5 minutes per wash) in TBS-T, the membrane was re-blocked for 1 hour and finally incubated with a different primary antibody.

2.3.4 PCR

PCR is a molecular biology technique used to amplify a single or a few DNA copies to billions of copies. It is based on the simple concept that it is possible to amplify a target region of DNA by incubating it with two short DNA fragments (the so-called primers) containing sequences complementary to the target region together with the appropriate enzyme responsible for DNA synthesis - DNA polymerase - from which the method takes its name[351]. As target DNA is amplified, it is used as a template for its replication causing an exponential amplification of the target DNA with each replication cycle. In general terms PCR consists of 30-40 cycles of replication each of them consisting of 1) a denaturation step during which double-stranded DNA is separated into single strands; 2) an annealing step during which the primers bind to the single-stranded DNA; 3) an extension step during which the DNA polymerase will synthesize a new DNA strand complementary to the DNA template.

2.3.4.1 RNA extraction

Total RNA was isolated from pellets using either the Qiagen RNeasy Mini Kit or the Applied Biosystems Arcturus Picopure RNA isolation kit (depending on sample size) according to the manufacturer's instructions. The quantity and purity of the extracted RNA was measured using a nanodrop spectrophotometer Nd-1000 (Labtech International, East Sussex, UK). An absorbance at 260nm quantified nucleic acid and the ratio of 260/280 determined purity (pure RNA ratio is 2.0).

2.3.4.2 cDNA synthesis

Reverse transcription of RNA into cDNA was carried out by using the High Capacity cDNA Reverse Transcription Kit. 10µL of the extracted RNA was mixed with 10µL of 2X Reverse Transcription Mastermix (2.2.4.8) in a PCR tube and reaction carried out as per manufacturer's protocol (i.e. 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds). cDNA was kept at -20°C for long-term storage.

2.3.4.3 Specific cDNA target amplification

In some experiments, due to low level of expression of target genes of interest, a preamplification step of the cDNA was carried out by using the TaqMan PreAmp mastermix kit. This step increases the quantity of specific cDNA targets for following gene expression analysis using TaqMan gene expression assays. Synthesized cDNA was incubated with a specific TaqMan PreAmp mastermix (2.2.4.11) containing a pool of primers (100 times diluted) specific to the target cDNAs which needed preamplifed. A preamplification reaction was carried as per manufacturer's protocol (i.e. 95°C for 10 minutes followed by 14 cycles consisting of 15 seconds at 95°C for denaturing and 4 minutes at 60°C for annealing and extension). The ensuing preamplified cDNA was diluted 1:5 in TE buffer and kept at -20°C for long-term storage.

In some experiments a one step reaction comprising all three above described steps was employed using the Invitrogen CellsDirect One-Step qRT-PCR kit as per manufacturer's protocol. Briefly 300 cells were directly sorted into 200µL PCR tubes containing single cell RNA extraction/cDNA/preamplification mastermix (2.2.4.12). The tubes were then vortexed to extract RNA and thereafter the following reaction conditions were set up in a thermal cycler: 50°C for 15 minutes for reverse transcription, 95°C for 2 minutes for Taq activation followed by 18 cycles of 95°C for 15 seconds and 60°C for 4 minutes for specific target amplification.

2.3.4.4 RT-PCR

RT-PCR is a method used to detect messenger RNA (mRNA) expression in a sample following its reverse transcription into cDNA. Following the PCR reaction, the PCR products are run on an agarose gel by electrophoresis and separated according to their size and stained with ethidium bromide prior to their detection. This is a qualitative analysis of mRNA expression within a sample and cannot be used to relatively quantify gene

expression levels between different samples. This is because ethidium bromide is a rather insensitive stain. Moreover the PCR products are usually analysed following their exponential stage of amplification (after 35-40 cycles usually), i.e. once the reaction is complete and has reached saturation. As a result the amount of PCR product has reached a plateau that is not directly correlated with the amount of target cDNA in the initial PCR.

First of all to carry out RT-PCR, custom primers were designed using NCBI/Primer-Blast. The cDNA sequences of the genes of interest were identified by searches on the Pubmed website and then used to design appropriate primers. Primer design was aimed to yield set of primers of about 20 base pairs with similar melting temperatures between forward and reverse primers, and no internal loops or primer dimers. The reaction was carried out by mixing synthesized cDNA with the RT-PCR Mix (2.2.4.9) in a PCR tube. The PCR reaction temperature and cycling conditions were optimised for each individual set of primers (Appendix I). The PCR products were run in 2% agarose gel in order to visualize individual DNA bands. The gel was prepared with 1g agarose powder in 50mL of 1X TBE buffer (2.2.4.7; 10X TBE buffer diluted in distilled water). This mixture was heated in a microwave until the agarose powder had completely dissolved. 1µL ethidium bromide (10mg/mL) was then added and the complete mix was poured into a casting tray with a comb inserted to create loading wells. The gel was left to set for about 30 minutes. Each well of the gel was loaded with 10µL of PCR product mixed with 2µL loading dye (6X) while 2µL of 100bp DNA ladder was loaded in a separate lane and then run at 50V in a tank filled with 1X TBE buffer. After running, the PCR products were detected using an ultraviolet light source (UV transilluminator - ChemiDoc).

2.3.4.5 qRT-PCR using TaqMan

qRT-PCR allows quantification of the cDNA products in a sample by detecting increasing level of fluorescence during the reaction as the PCR products accumulate, i.e. in real time. In a real-time PCR reaction a fluorescent reporter molecule such as a TaqMan Probe or SYBR Green dye, is added to the reaction mix and used to monitor the accumulation of PCR product. As a result the amount of fluorescence emitted from the reporter increases as the quantity of target cDNA increases. By plotting fluorescence against the number of cycles of the PCR reaction it is possible to determine the cycle number at which the increase in fluorescence (and therefore cDNA) is exponential and exceeds the set background fluorescence threshold. This is called the cycle threshold or Ct value for a specific PCR reaction. By recording the Ct value during the exponential phase of the

reaction it is possible to infer the quantity of the cDNA in a sample relative to each other. This is because during its exponential phase, the reaction has not yet reached its saturation and the DNA target doubles every cycle so that the quantity of the PCR product is directly proportional to the amount of template nucleic acid with any differences in fluorescence signal secondary to different starting amounts of cDNA in the samples tested. Simply put as an example, a DNA sample whose Ct precedes that of another sample by 3 cycles contained $2^3 = 8$ times more template for the reaction.

TaqMan probes consist of a reporter fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. The quencher molecule quenches the fluorescence emitted by the reporter fluorophore and, as long as the reporter and the quencher are in proximity, inhibits any fluorescence signals. The probes are designed to anneal within the DNA region amplified by the specific set of primers they are associated with. During the PCR reaction the probe is cleaved by the 5' to 3' exonuclease activity of the Taq polymerase causing physical separation of the reporter and the quencher dye. This results in an increase in fluorescence as the PCR products accumulate. The advantage of the TaqMan Probe as a fluorescent reporter is that the probe set is complementary to the target amplicon. The increase in fluorescent signal occurs therefore only if the target sequence complementary to the probe is amplified during PCR. Consequently nonspecific amplification such as primer dimers, which are a potential problem when using SYBR Green dye as a fluorescent reporter, is not detected (figure 2-6).

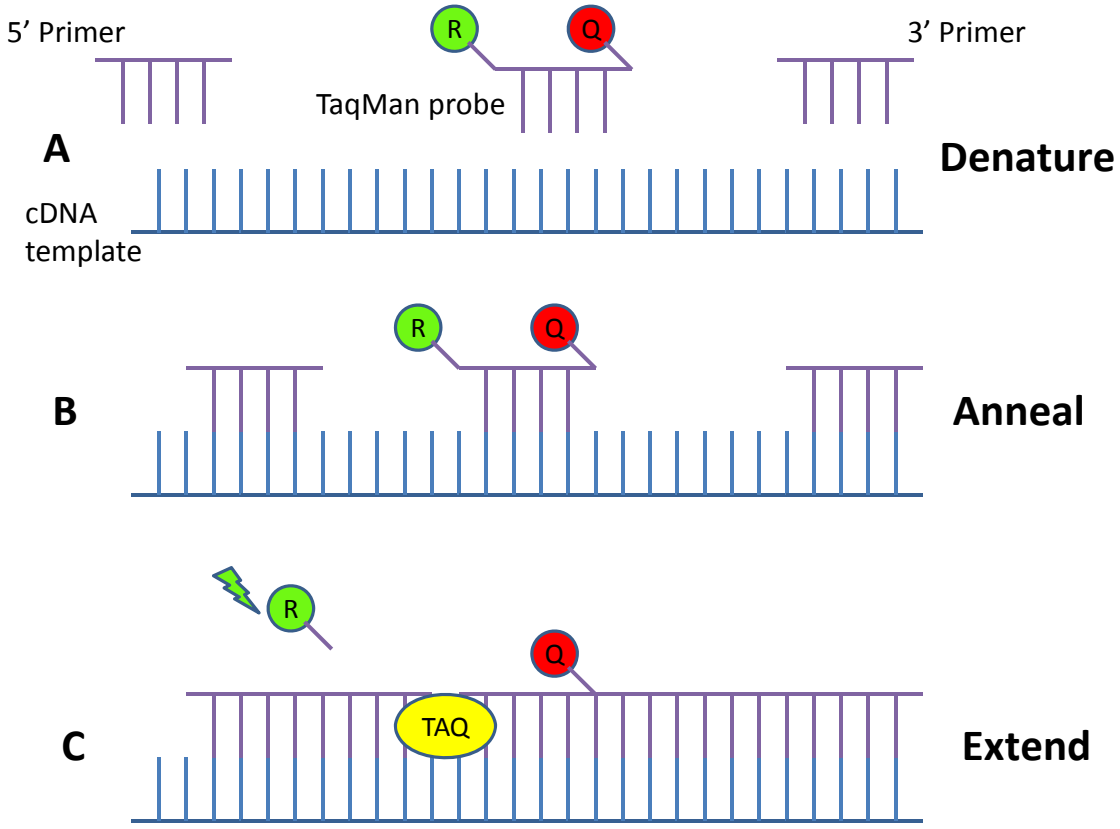


Figure 2-6 Schematic diagram for the mechanism of function of the TaqMan qRT-PCR During the denaturation step of the PCR (A) the probe and primers are not attached to the cDNA template, the reporter (R) and quencher (Q) fluorophore are in close proximity and no fluorescence is emitted. During the annealing step (B) the probe and primers bind to the cDNA template but the reporter (R) and quencher (Q) fluorophore are still in close proximity and no fluorescence is emitted. However during the extension step (C) the TAQ polymerase adds complementary nucleotides to the single strand cDNA template and also cleaves the TaqMan probe thus releasing the reporter (R) from the quencher (Q) fluorophore and allowing for fluorescence to be emitted.

Practically the mRNA levels of the target genes were measured using the ABI PRISM 7900HT sequence detector (Applied Biosystems (ABI), U.K.). cDNA was added to the TaqMan qRT-PCR master mix (2.4.4.10) in triplicate reactions and run on the ABI PRISM 7900 with the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Experimental target quantities were normalized to the endogenous control using $2^{-\Delta CT}$ method for each individual samples. For matched samples (i.e. treated versus UT) the relative quantification (RQ) of gene expression was calculated using the comparative CT method, i.e. $2^{-\Delta\Delta CT}$[352].

2.3.4.6 qRT-PCR using Fluidigm

The Fluidigm BioMark HD System integrates thermal cycling and fluorescence detection on a Fluidigm dynamic array integrated fluidic circuit (IFC) and provides high-throughput qRT-PCR using standard TaqMan gene expression assays[353]. The IFC allows the

simultaneous performance of PCR reactions in nL volumes on a single microfluidic device. The BioMark HD System consists of a real-time PCR instrument (BioMark HD Reader) and an IFC controller to prime and load the IFC.

Briefly, cDNA was subjected to a specific targets amplification step (2.3.4.3) prior to performing the PCR reaction. Thereafter 48 qRT-PCR sample mixes (2.2.4.14) and 48 qRT-PCR assay mixes (2.2.4.13) were loaded into the wells of a previously primed 48x48 dynamic array IFC. The qRT-PCR sample and assay mixes were combined (by the IFC controller) through the IFC chip network of microfluidic channels, chambers and valves automatically assembling a total of 2304 (48x48) individual PCR reactions. Thereafter the loaded chip was put through thermal cycling within the BioMark HD Reader and a Ct value for each individual PCR reaction was produced.

The actual mechanism of qRT-PCR and the calculation of relative expression of target genes were the same as previously described for qRT-PCR using TaqMan (2.3.4.5).

2.3.5 Statistical analysis

All the results are shown as the mean ± standard error of the mean (mean ± SEM) unless otherwise stated. All statistical analyses was performed with Graph Pad prism software using the two-sided unpaired or paired student's t-test (depending on the nature of samples compared) when two groups were compared. When multiple groups were compared a one way ANOVA with correction for multiple groups' comparison was used. A p value <0.05 was considered significant.

3 Results (I) Assessment of autocrine GF production by CML SPC and normal HSPC

Autocrine production of GFs is a mechanism used by various types of cancer to promote their survival and proliferation. It has been described both in solid and haematological malignancies[354,355] and shown to be relevant to the survival and resistance to therapy of CSC in other forms of cancer[356].

Autocrine production of myeloid haemopoietic GFs by CML SPC (IL-3, G-CSF) or MNC (GM-CSF) has already been demonstrated[262,270] and this autocrine loop is thought to contribute to the GF independent growth capacity of CML SPC and possibly also to BCR-ABL induced transformation (fully reviewed in section 1.3.6.2). Moreover other GFs, whose biological function in myeloid cells development is less obvious, such as IL-4, vascular endothelial growth factor (VEGF) and insulin like growth factor 1 (IGF-1)[271,357,358] have been shown to be produced by immortalised BCR-ABL positive cells. However little is known regarding the production of the latter GFs by primary CML SPC and their potential biological function and role in this context.

It has already been shown that CML SPC can activate several survival and antiapoptotic signalling pathways following exposure to haemopoietic GFs, even in the presence of TKI[261,263]. It is therefore intriguing to postulate that autocrine cytokine overexpression could also act as an early resistance mechanism accounting for CML SPC survival following TKI selection pressure. Extending therefore the current knowledge of the autocrine GF production by CML SPC could lead to the identification of novel survival mechanisms of CML SPC which could in turn be therapeutically targeted. In this respect it should be noted that a certain level of autocrine GF production has been shown in normal HSPC[34] and therefore caution has to be exercised in interpreting autocrine GF production as a cancer specific feature. This observation has obvious implications on the safety of a therapeutic approach aimed to target GF production or signalling in CML.

Finally another important and still unanswered question is if autocrine GF production in CML SPC is dependent on BCR-ABL kinase activity.

Based on this brief summary of our knowledge to date, the aims set out in this chapter were to:

1) extend our knowledge of which GFs and cognate receptors are differentially expressed between CML LSCs ($CD34^+$ $CD38^-$) and LPCs ($CD34^+$ $CD38^+$) relative to their normal counterparts.

2) elucidate if GF expression is modulated by BCR-ABL kinase activity.

First the expression levels of several GFs was measured and compared between normal HSPC and CML SPC. QRT-PCR was used in the first instance to detect their expression because of its high sensitivity. This decision was taken because autocrine produced GFs are often expressed at low levels which would make their detection by other techniques, such as ELISA or western blotting, more difficult, and taking into account the limited material available when working with primary human cells. However for the most interesting candidates, confirmation of their expression at the protein level was also sought.

Thereafter to assess if any residual autocrine GF production was present upon complete BCR-ABL kinase inhibition, primary CML SPC were treated with a high concentration of NL at 5μM prior to analysing their expression of GFs and cognate receptors. NL was deliberately chosen as being a more potent TKI than IM and more specific than DA, while the high concentration (which is slightly above peak plasma concentrations achievable in patients[359]) has been shown to achieve maximal (nearly 100%) inhibition of BCR-ABL kinase activity[263].

This was a crucial point based on recent evidence which has shown that CML LSCs do not require BCR-ABL kinase activity for their survival[337,338]. Assuming that autocrine GF production is a survival mechanism for CML LSCs, it would be important to understand if it is a BCR-ABL kinase independent mechanism. Only in this case could it be regarded as a *bona fide* therapeutic target worthy of further investigation.

3.1 Assessment of GFs and cognate receptors expression in CML SPC and normal HSPC by PCR

3.1.1 Initial screen for autocrine GFs expression in BCR-ABL positive cell lines and CML CD34$^+$ cells by RT-PCR

Preliminary experiments to assess autocrine GF expression in the BCR-ABL positive cell lines, K562 and KCL22 were performed to first screen for the most interesting candidates and identify those to be followed in subsequent experiments using primary human CML cells. Specific sets of primers were designed for several GFs, including *IL-2*, *IL-4*, hepatocyte growth factor (*HGF*), *GM-CSF*, *SCF*, *TGF-β1* and *TNF-α*, chosen on the basis of their known functions as regulators of different stages of haemopoiesis and/or because they had already been implicated in autocrine signalling in other malignancies, but had not already been shown to be produced by CML SPC. After optimisation of the best cycling conditions to test each set of primers (Appendix I), expression of the GFs was assessed by standard RT-PCR.

Amongst all GFs tested, gene expression for *TGF-β1*, *IL-4* and *GM-CSF* was consistently demonstrated in K562 cells, while TNF-α and HGF were consistently detected in KCL22 cells (figure 3-1 A and B). No products were detected for *IL-2* or *SCF*.

Overall these data demonstrate mRNA expression of novel GFs by BCR-ABL positive cell lines thus providing support for testing if production of the same is present in CP CML CD34$^+$ cells.

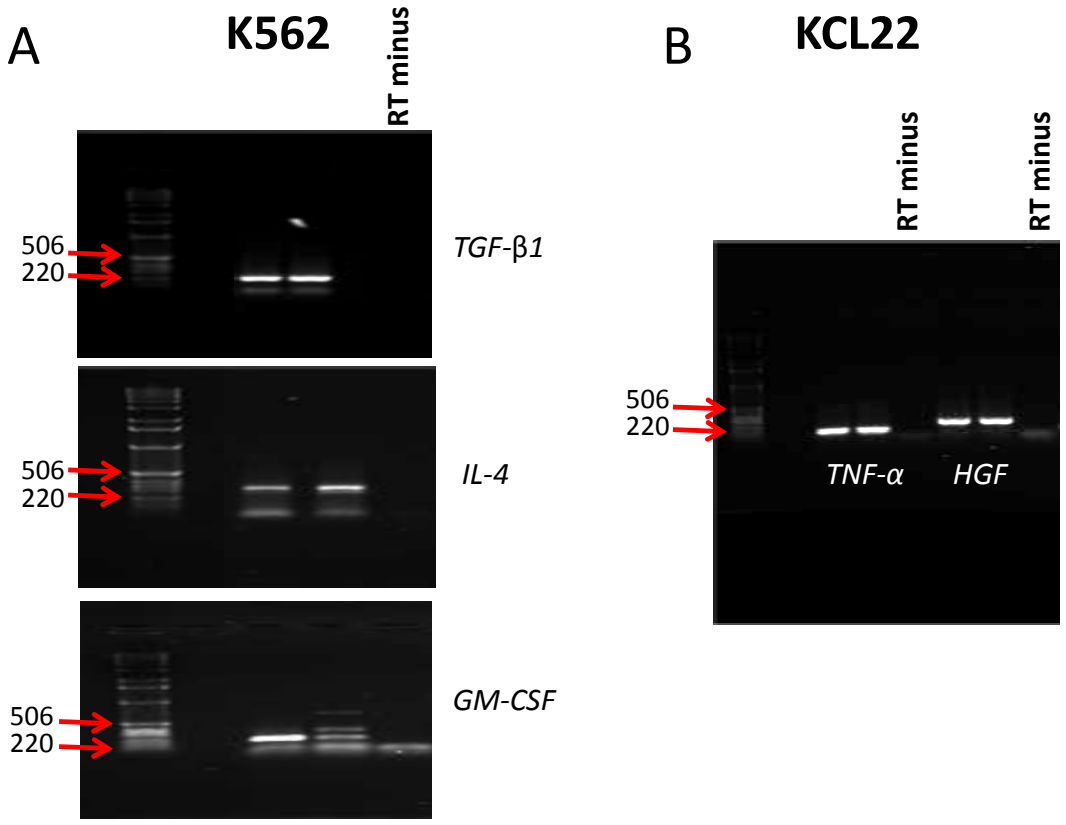


Figure 3-1 GF expression in BCR-ABL positive cell lines
Candidate GF mRNA expression was measured by RT-PCR in K562 (A) and KCL22 (B) following resuspension in fresh medium and overnight culture (n=2). RT minus, no reverse transcriptase control. Red arrows indicate DNA ladder marks with numbers referring to the basepairs number of the mark.

GF expression was therefore tested in primary CML $CD34^+$ cells by standard RT-PCR. MNC from normal healthy volunteers were also tested as positive controls as they are rich in lymphocytes and monocytes known to produce several of these GFs. While *TNF-α*, *TGF-β1*, *HGF* were easily detected in both $CD34^+$ cells and MNC, *IL-2*, *IL-4* and *GM-CSF* were only detected in the MNC (figure 3-2 A and B). However following a preamplification (2.3.4.3) step, *IL-4*, and *GM-CSF* became detectable in 2 out of 3, and 1 out of 3 CML patients $CD34^+$ cells, respectively (figure 3-2 C).

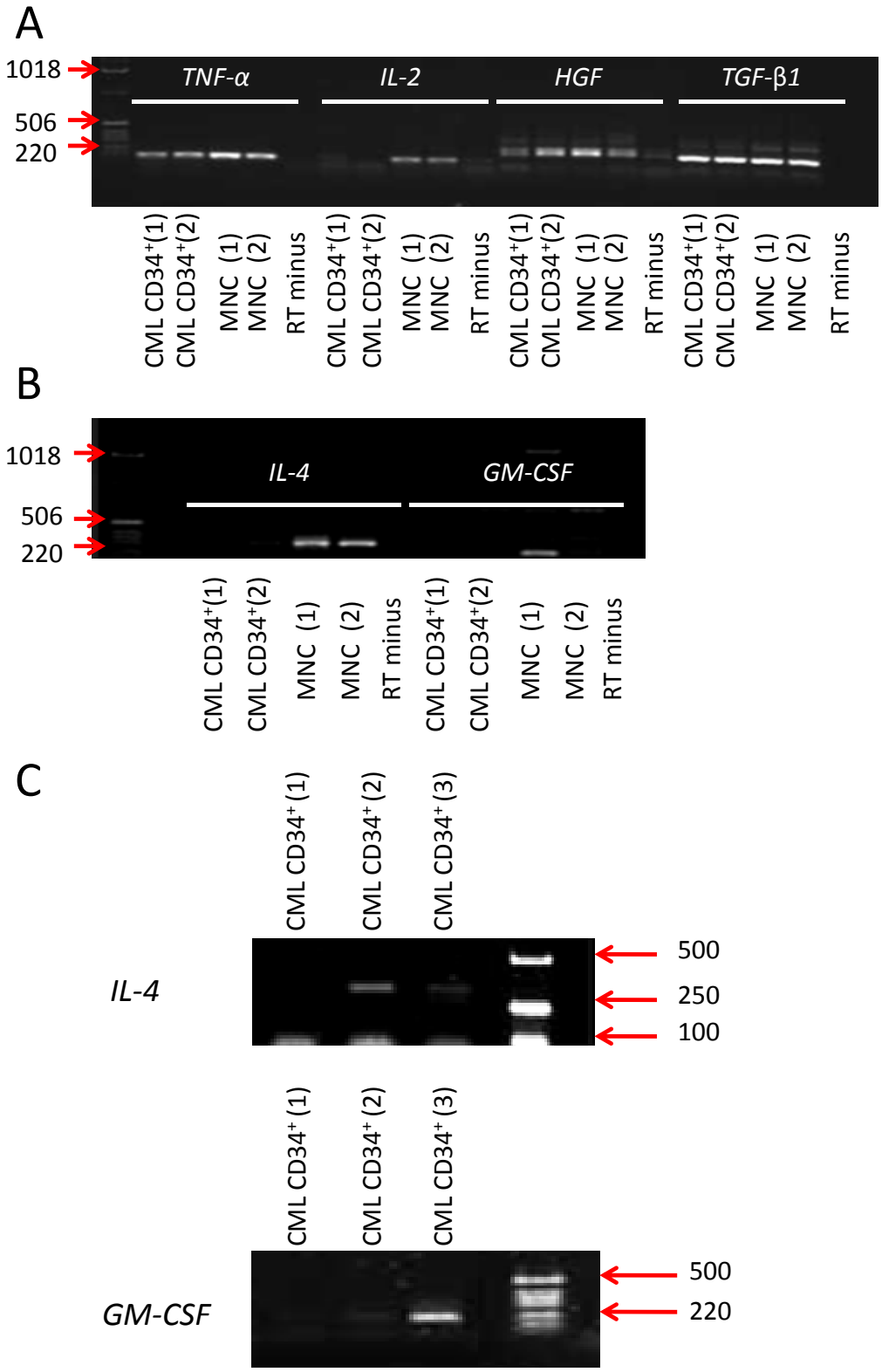


Figure 3-2 GF expression in CML CD34$^+$ cells
mRNA expression of GFs was measured in 2 CML CD34$^+$ cells following recovery and overnight culture in SFM+PGF cells and freshly isolated MNC (used as a positive control) by standard RT-PCR (panels A and B). Detection of *IL-4* and *GM-CSF* mRNA expression in 3 CML CD34$^+$ samples following a cDNA preamplification step (panel C).

Overall these results identify novel GF expression by primary CML CD34$^+$ cells, such as TGF-β1, TNF-α, IL-4 and HGF. The next logical step was to accurately quantify levels of GF expression in CML CD34$^+$ versus normal CD34$^+$ cells using TaqMan qRT-PCR.

3.1.2 Assessment of levels of GF expression in CML and normal CD34$^+$ cells by qRT-PCR

Quantitation of mRNA expression for *GM-CSF*, *HGF*, *IL-4*, *TGF-β1* and *TNF-α* was performed in CML and normal CD34$^+$ cells. A preamplification step (2.3.4.3) was carried out to improve GF detection given their low levels of expression and the limited amount of RNA normally obtained from primary CD34$^+$ cells. Between 5 and 13 CML samples and 5 to 6 normal samples were analysed for expression of candidate GFs.

Amongst the candidates both *TNF-α* and *IL-4* appeared to be more highly expressed in CML CD34$^+$ compared to normal CD34$^+$ cells. However due to the high inter-sample variability, the differences did not reach statistical significance (respectively p=0.16 and p=0.2) (figure 3-3 A and B). *GM-CSF* was detected in a higher proportion of CML CD34$^+$ (8 out 10, 80%) compared to normal CD34$^+$ samples (4 out 6, 66.6%), but no significant difference in the expression levels was noted due to high inter-sample variability (figure 3-3 C). Given the previously reported autocrine production of IL-3 by CML CD34$^+$ cells[270] and the observation that *GM-CSF* was more easily detected in CML versus normal CD34$^+$ cells, the mRNA levels of the common β chain receptor of IL-3 and GM-CSF (*CSF2RB*) were also measured. Interestingly *CSF2RB* was more highly expressed in CML CD34$^+$ cells at a level approaching statistical significance (p=0.09) (figure 3-3 D). Finally there was no obvious difference in the expression levels of *TGF-β1* between CML and normal CD34$^+$ cells, while *HGF* appeared to be more highly expressed in normal CD34$^+$ cells, but the difference did not reach statistical significance (p=0.11) (figure 3-3 E and F).

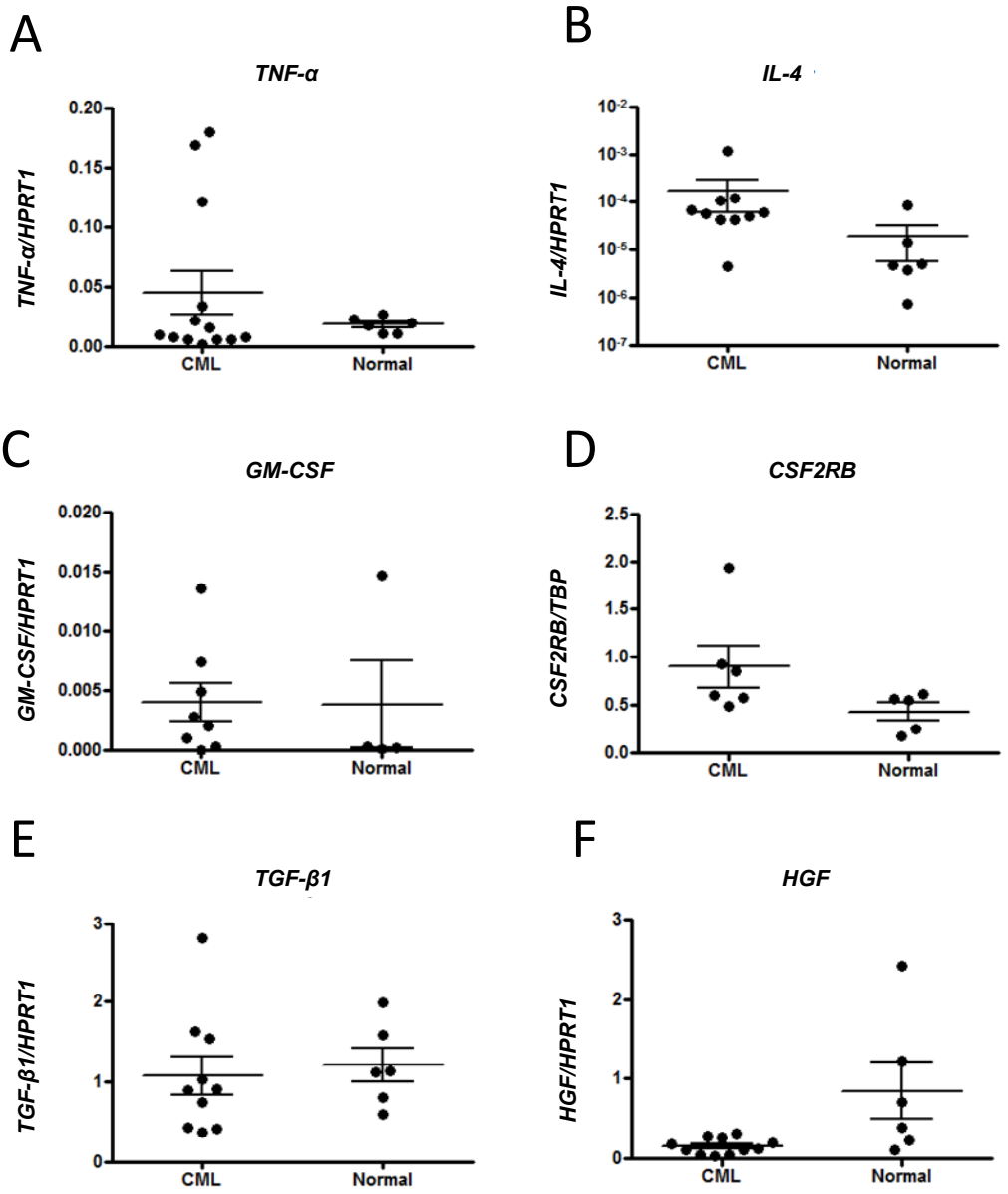


Figure 3-3 Quantitative mRNA expression of candidate GFs in CML and normal CD34$^+$ cells
mRNA expression of GFs was measured in CML (n= 6 to 13) and normal CD34$^+$ (n=6) samples following recovery and overnight culture in SFM+PGF. Levels of expression were calculated using the $2^{-\Delta Ct}$ method after normalisation of GFs mRNA expression levels against the expression levels of a housekeeping gene (*HPRT1* or *TBP*) within each sample.

Overall these data suggest an increased expression of some of the candidate GFs and one of their cognate receptors in CML CD34$^+$ cells compared to their normal counterparts, but not to a statistically significant level. It should be noted however that most of these GFs are also normally produced at higher levels by monocytes and macrophages (*TNF-α*), lymphocytes (*IL-4* and *GM-CSF*), endothelial and stromal cells (*GM-CSF*). Although all samples used were enriched for CD34$^+$ cells (to 85% to 100% depending on the samples) an effect on the levels of GFs expression due to contaminating MNC cannot be ruled out, which makes an accurate measurement of the levels of GFs production by both normal HSPC and CML SPC using enriched CD34$^+$ samples potentially not completely accurate.

In view of this it was decided to assess GF mRNA expression in sorted population looking at both the more primitive stem cell population ($CD34^+$ $CD38^-$) and the more mature progenitor population ($CD34^+$ $CD38^+$). This approach provided a more accurate measurement of the levels of expression of GFs in CML LSCs, CML LPCs and normal counterparts while reducing the contribution of any contaminating non haemopoietic cells and MNC. It also allowed characterisation of any differential expression of GFs between the more mature progenitor compartment ($CD34^+$ $CD38^+$) and the primitive stem cell compartment ($CD34^+$ $CD38^-$) which in CML samples is enriched for cells resistant to TKI.

3.1.3 Assessment of levels of GF expression in CML and normal $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ cells by qRT-PCR

Levels of expression for the same GFs previously measured in the bulk $CD34^+$ cells were measured in freshly sorted $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ samples as previously described (2.3.1.5). Interestingly following this further purification step, expression of *IL-4* became undetectable in the $CD34^+$ $CD38^-$ compartment of the 5 CML and 3 normal samples tested, while its expression was still detected, although at low level, in the $CD34^+$ $CD38^+$ CML cells, but not in their normal counterparts (only 1 out of 3 samples) (figure 3-4 A). Similarly *GM-CSF* was only detected in CML but not in normal samples within both compartments, although expression levels were quite variable and undetectable in 2 out of the 5 CML samples tested (figure 3-4 B). *TNF-α* was instead detected at a similar level in both $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ CML cells; following sorting it was expressed at significantly higher levels in both CML populations compared to normal counterparts ($p=0.03$) (figure 3-4 C). *CSF2RB* was significantly more expressed in CML $CD34^+$ $CD38^+$ versus CML $CD34^+$ $CD38^-$ cells ($p=0.008$). Moreover it was also more highly expressed in both CML compartments compared to their normal equivalents, although the difference reached statistical significance only in the comparison between the $CD34^+$ $CD38^+$ compartments ($p=0.04$ for $CD34^+$ $CD38^+$ and $p=0.2$ for $CD34^+$ $CD38^-$) (figure 3-4 D).

HGF and *TGF-β1* were also easily detected. Both *TGF-β1* and *HGF* were more highly expressed in the normal $CD34^+$ $CD38^-$ cells compared to the CML counterparts, with the difference reaching statistical significance for *HGF* (respectively, $p=0.07$ and $p=0.008$). Moreover *HGF* was also differentially expressed between the 2 CML compartments with significantly higher levels detected in the $CD34^+$ $CD38^+$ cells ($p=0.04$) (figure 3-4 E-F).

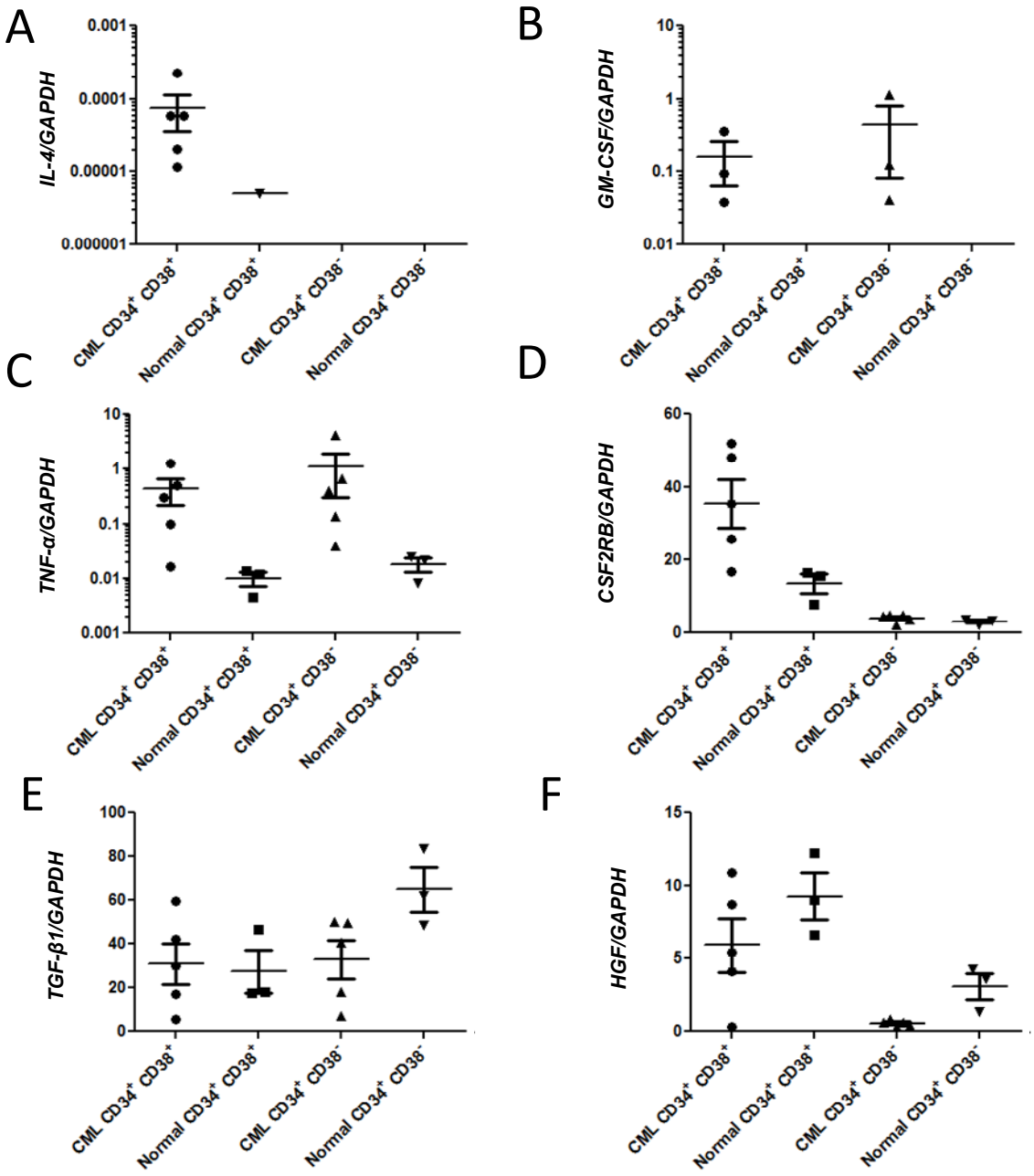


Figure 3-4 Quantitative mRNA expression of candidate GFs in sorted CML and normal $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ cells
mRNA expression of GFs was measured in CML (n=5) and normal (n=3) samples following recovery, sorting into $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ cells and overnight culture in SFM+PGF. Levels of expression were calculated using the $2^{-\Delta Ct}$ method after normalisation within each sample of GFs mRNA expression levels against the expression levels of a housekeeping gene (*GAPDH*).

Overall these data suggest that once expression levels are measured within populations enriched for LPCs and LSCs, differences are more easily detected and start to be significant with higher levels of expression for some GFs and receptors seen in both CML LSCs (*CSF2RB*, *GM-CSF* and *TNF-α*) and CML LPCs (*CSF2RB*, *GM-CSF*, *IL-4* and *TNF-α*) compared to normal and vice versa (*HGF*). Based on these data, it was decided to focus mainly on the GFs expressed at higher levels by CML SPC.

3.1.4 Assessment of levels of GF expression in CML CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells upon BCR-ABL kinase inhibition by qRT-PCR

To assess the effects of BCR-ABL kinase inhibition on gene expression of GFs, CML CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells were sorted and cultured in SFM+PGF in the presence of NL at a concentration of 5μM. Twenty four hours duration of NL treatment prior to the measurement of GFs expression was chosen following preliminary experiments in CML CD34$^+$ cells. The experiments comprised analysis by western blotting of p-CrKL, a known downstream target of BCR-ABL and surrogate marker for its kinase activity, showing that BCR-ABL kinase was effectively inhibited within 1 hour of treatment with NL at 5μM and such effect was persistent up to 24 hours (figure 3-5 A). Moreover using annexin-V staining, only low levels of apoptosis (similar to baseline level) were detected in CML CD34$^+$ cells treated with 5μM of NL at 24 hours (figure 3-5 B). Therefore the 24 hours time point was considered as an appropriate time to assess modulation of GF expression by BCR-ABL kinase because of the evidence that persistent inhibition of BCR-ABL kinase activity was achieved without inducing high apoptosis levels and based on the reasonable assumption that gene expression changes would have started to occur by then.

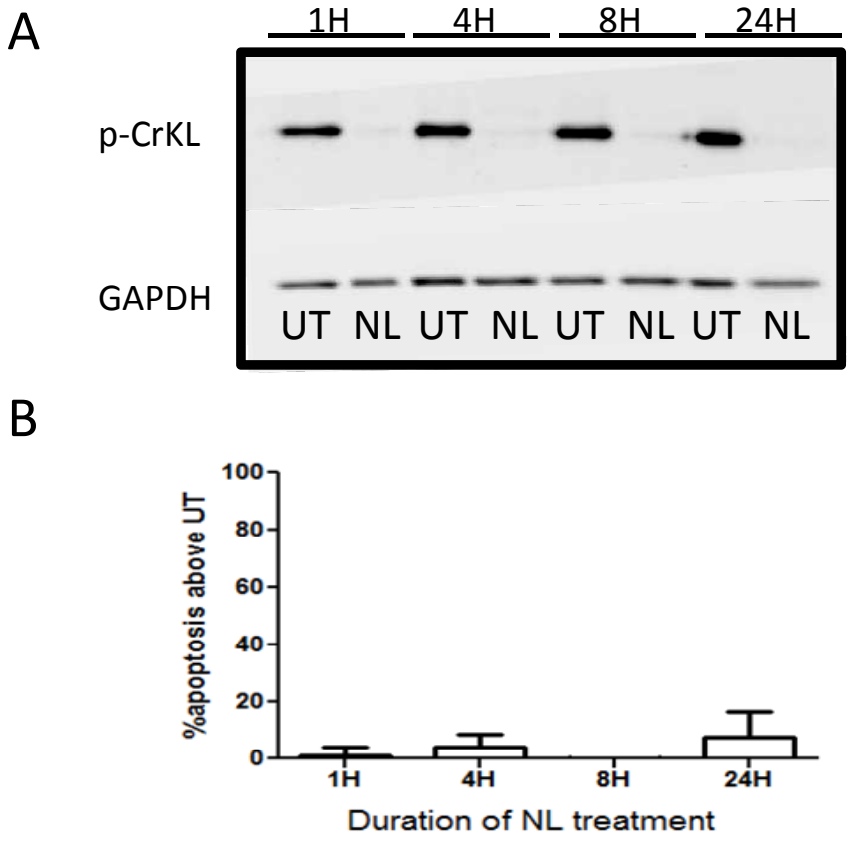


Figure 3-5 P-CrKL levels and apoptosis induction following NL treatment in CML CD34$^+$ cells
P-CrKL levels were measured by western blotting in CML CD34$^+$ cells following 5μM NL treatment at 1, 4, 8 and 24 hours and compared to UT control levels (panel A, n=2). Apoptosis levels were measured by annexin-V staining in CML CD34$^+$ cells following 5μM NL treatment at 1, 4, 8 and 24 hours (panel B, n=5) and compared to those of UT cells.

Therefore CML CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells were treated with 5μM NL for 24 hours and GF expression measured by qRT-PCR (figure 3-6 A and B)

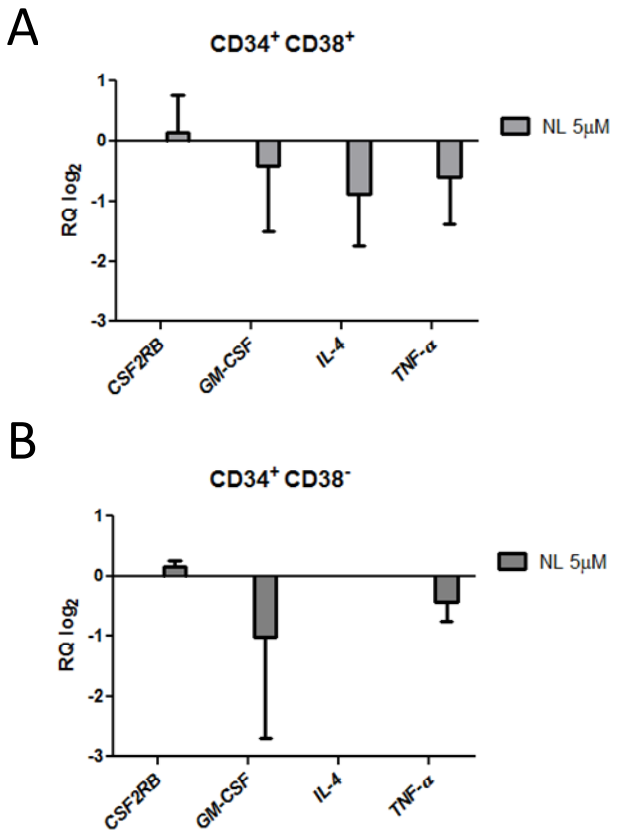


Figure 3-6 Quantitative mRNA expression of candidate GFs in CML CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells following NL treatment
GF expression was measured in CML samples (n=3) following sorting into CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells and overnight culture in SFM+PGF with or without NL at 5μM. Differences in GF expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of GFs mRNA expression levels against the expression levels of a housekeeping gene (*GAPDH*). RQ, i.e. positive or negative fold change, of GFs mRNA expression following NL treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the untreated cells having a value of 0 in the graph).

Interestingly no changes in gene expression levels were detected for *CSF2RB* while *TNF-α* expression levels were only marginally reduced (less than 50%) following NL treatment in both CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells. Slightly higher levels of modulation were observed for *GM-CSF* mainly within the CD34$^+$CD38$^-$ compartment and *IL-4* (for the latter GF only for the CD34$^+$ CD38$^+$ cells as it was not detected in the CD34$^+$CD38$^-$ cells). However due to sample variability none of these differences was statistically significant. Overall, these results suggest that gene expression of GFs and cognate receptors is not significantly modulated by BCR-ABL kinase activity.

3.2 Assessment of GFs and cognate receptors protein expression

3.2.1 Assessment of levels of TNF-α and CSF2RB protein expression in CML and normal $CD34^+$ cells

Following demonstration of differential expression at mRNA levels between CML and normal SPC for *CSF2RB*, *GM-CSF*, *IL-4* and *TNF-α*, the next logical step was to analyse if this translated into a differential level of expression at the protein level. Given their very low and inconsistent levels of mRNA expression, GM-CSF and IL-4 were not easily detectable and accurately measured. However for CSF2RB and TNF-α, it was possible to accurately measure the levels of protein expression within the $CD34^+$ cells by flow cytometry analysis.

Protein analysis was performed on cells following 48 hours in culture with SFM+PGF. This longer culture period was chosen to allow enough time for the differential gene expression levels detected at the earlier time point of 24 hours to translate into differential expression at the protein level. Given the longer culture period of these experiments, the protein expression analysis was performed on a pure $CD34^+$ population gated by flow cytometry, but not on subpopulations. The CD38 marker tends to be expressed following *in vitro* culture making an assessment within subpopulations challenging and possibly unreliable. However by gating on a pure $CD34^+$ population any contribution of MNC to the protein levels measured was effectively eliminated.

Flow cytometry analysis confirmed higher levels of protein expression for both TNF-α and CSF2RB in CML $CD34^+$ versus normal $CD34^+$ cells. In particular TNF-α protein expression levels were twice as high in CML versus normal $CD34^+$ cells ($p=0.038$) (figure 3-7 A), while CSF2RB protein expression levels were 1.5 times higher in CML $CD34^+$ cells ($p=0.032$) (figure 3-7 B).

Overall these results further confirm at the protein expression level the findings previously obtained at the gene expression level.

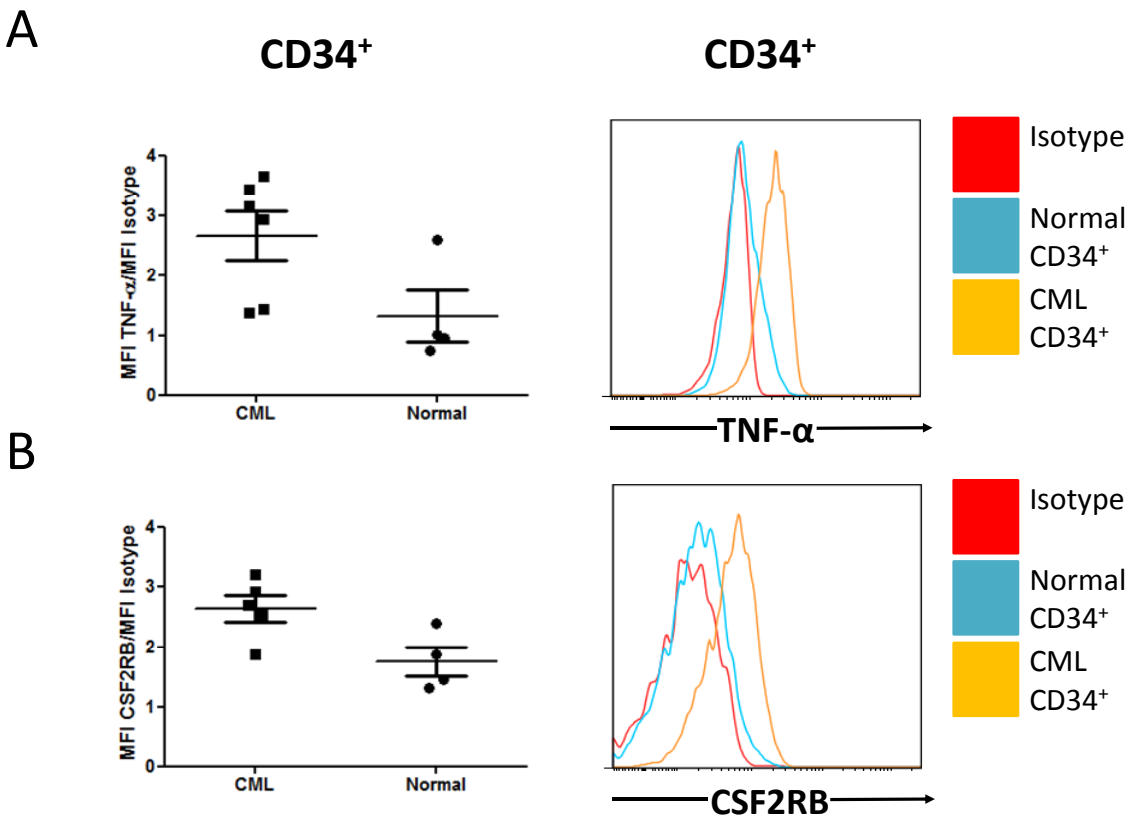


Figure 3-7 Protein expression levels of TNF-α and CSF2RB in CML and normal CD34$^+$ cells
CD34$^+$ cells from CML (n=6) and normal (n=4) samples were cultured for 48 hours following recovery and protein expression was then measured by flow cytometry. Levels of expression were expressed as MFI of the antibody stained cells over MFI of cells stained with a matched isotype antibody (left panels). Representative flow plots are shown in the right panels.

3.2.2 Assessment of levels of TNF-α and CSF2RB protein expression in CML CD34$^+$ cells upon BCR-ABL kinase inhibition

Finally the effect of BCR-ABL kinase inhibition on the protein expression of TNF-α and CSF2RB by CML CD34$^+$ cells was investigated. CML CD34$^+$ cells were therefore cultured for 48 hours in the presence or absence of NL at 5μM and thereafter TNF-α and CSF2RB protein expression was measured.

Consistent with the gene expression data TNF-α protein expression following NL treatment was only reduced by about 20% with the difference not reaching statistical significance (p=0.33) (figure 3-8 A). However for CSF2RB a more pronounced reduction by about 40% in protein expression levels was observed with the difference bordering statistical significance (p=0.06) (figure 3-8 B). This latter result was not in keeping with the gene expression data, thus suggesting that the levels of CSF2RB might be regulated at a post-translational level following BCR-ABL kinase inhibition, for example as a result of an increased protein degradation. Alternatively the longer 48 hours exposure prior to the assessment of protein expression might have elicited an effect which was not detected by the gene expression analysis carried out at the earlier time point of 24 hours.

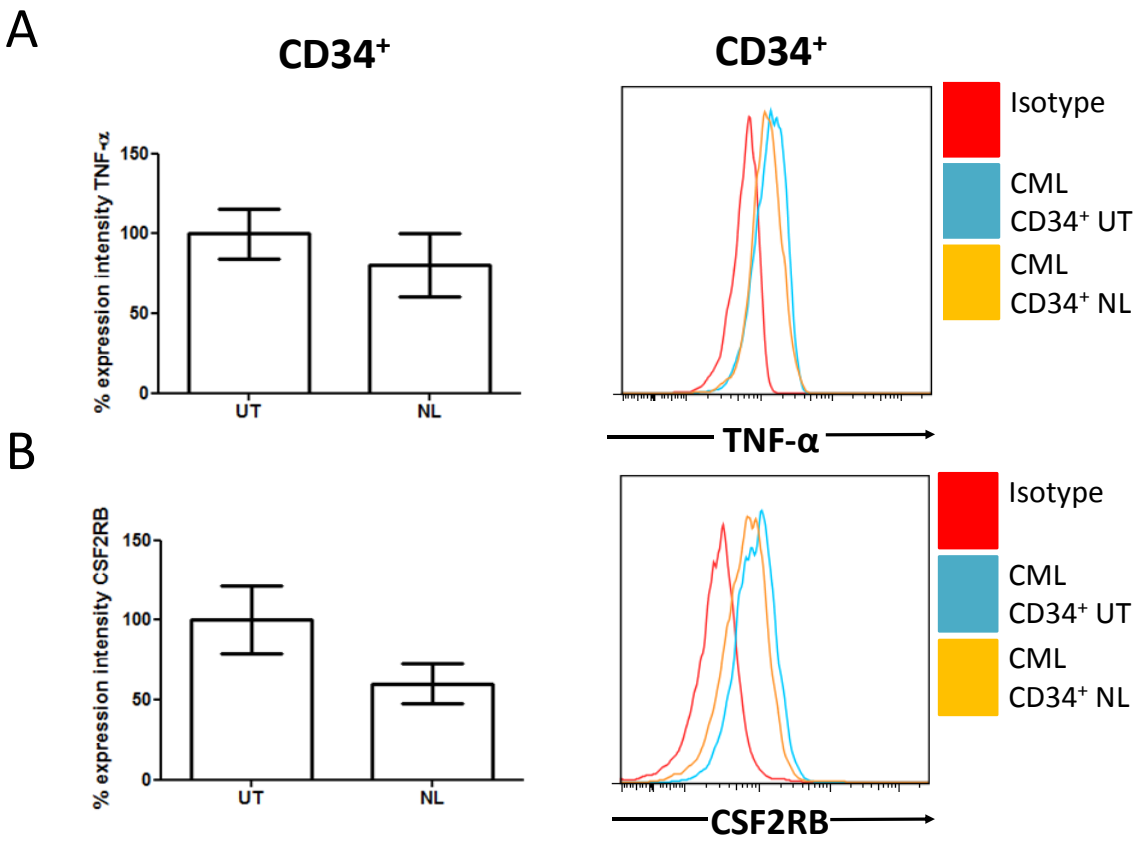


Figure 3-8 Protein expression levels of TNF-α and CSF2RB in CML CD34$^+$ cells upon BCR-ABL kinase inhibition
Levels of expression were measured by flow cytometry analysis and expressed as MFI of the antibody stained cells over MFI of cells stained with a matched isotype antibody (n=4). The average expression levels in the NL treated cells were then plotted as percentage of the average expression levels in UT cells (left panels). Representative flow plots (right panels).

3.3 Summary and future directions

The autocrine production of GFs by cancer cells has long been postulated as a mechanism beneficial to their survival and proliferation. In CML, however, the known profile of GFs produced by the leukaemic SPC was limited to IL-3 and G-CSF[270] and the exact role played by BCR-ABL kinase in the regulation of this event was never investigated. The purpose of the work presented in this chapter was to extend the current knowledge of the GFs expressed by CML SPC, determine if their level of expression was different from the levels detected within normal $CD34^+$ cells and finally assess the effects of BCR-ABL kinase inhibition on this phenomenon. Starting from BCR-ABL positive cell lines it was found that several novel GFs are produced by CML cells, including CML SPC, in an autocrine fashion, such as *GM-CSF*, *HGF*, *IL-4*, *TGF-β1* and *TNF-α.* When however the levels of expression in CML $CD34^+$ and normal $CD34^+$ cells were quantified using qRT-PCR even more interestingly it appeared that many of these were also produced by normal $CD34^+$ cells. Autocrine production of some of these GFs (namely *HGF*, *TGF-β1* and *TNF-α*) by normal $CD34^+$ cells has already been reported and therefore these findings are consistent with published literature[34]. Also consistent with the same report, other GFs such as *GM-CSF* were barely detected in normal $CD34^+$ compared to CML $CD34^+$ cells. A trend towards increased expression of some GFs and their receptors (*TNF-α*, *IL-4*, *CSF2RB* and *GM-CSF*) was seen in CML $CD34^+$ compared to normal $CD34^+$ cells, however these differences were not statistically significant. Although the samples used for this analysis were highly enriched for $CD34^+$ positive cells (between 85% to 100%), in order to exclude any contribution from contaminating MNC in the results observed, phenotypically purified sorted population of normal and leukaemic stem ($CD34^+$ $CD38^-$) and progenitor ($CD34^+$ $CD38^+$) cells were tested for the expression of the same GFs. This had also the advantage to measure the levels of autocrine production of these same GFs within different subsets of SPC, which might help to postulate their different biological relevance. Interestingly once the analysis was carried in purified sorted populations statistically significant differences were observed for *CSF2RB* and *TNF-α* with higher levels in CML compared to normal cells. Low levels of expression of *GM-CSF* and *IL-4* were detected only in CML cells, with generally higher levels in the more mature $CD34^+$ $CD38^+$ compartment. It is worth noting that the autocrine expression levels of the more typical myeloid GFs, such as *GM-CSF* and its receptor *CSF2RB* (the common GM-CSF and IL-3 β chain receptor), whose function is more relevant in proliferation and differentiation of myeloid precursors, were higher in CML $CD34^+$ $CD38^+$ cells relative to CML $CD34^+$ $CD38^-$ cells. This observation is biologically consistent with previous published data showing that primitive quiescent

cells (likely to be enriched in $CD34^+$ $CD38^-$ cells) do not produce autocrine *IL-3* until they enter cell cycle[342] and would suggest that the effects of some of these autocrine GFs, mainly those involved in myeloid cells maturation, might be specific to the survival and proliferation of more mature cells while having a limited role on more primitive stem cells. Conversely the higher levels of expression of *CSF2RB* in all CML compartments compared to normal might still allow CML LSC compared to normal HSC to be exposed to enhanced prosurvival signals through paracrine produced GFs. Moreover the previously reported interaction of CSF2RB with BCR-ABL oncoprotein leading to its ligand independent activation[274] and constitutive activation of JAK2 suggests that indeed CSF2RB might have an important role in relaying enhanced survival and proliferation signals in BCR-ABL positive cells. Because of its higher expression and putative interaction with BCR-ABL, CSF2RB might therefore be a legitimate therapeutic target, either on its own or through its downstream activated kinase, namely JAK2.

A limitation of these investigations was the fact that confirmation at the protein level of the expression of the aforementioned GFs was not always possible, namely for IL-4 and GM-CSF. This was likely secondary to their low expression levels in primary cells which made their detection difficult as also previously reported for normal $CD34^+$ cells[34]. However, using flow cytometry analysis, protein expression was detected at least for CSF2RB and TNF-α. Reassuringly the protein expression data for both TNF-α and CSF2RB were consistent with gene expression data showing higher levels of expression in CML $CD34^+$ compared to normal $CD34^+$ cells, which is suggestive that the gene expression data might mirror the protein expression levels in the other instances too. However it has to be noted that by using flow cytometry analysis for protein expression, the actual secretion of the GFs was not demonstrated which represents another limitation of the current data.

One important question in regards to autocrine production of GFs by BCR-ABL positive cells which had never been addressed before is whether or not this phenomenon is dependent on BCR-ABL kinase activity. Using a 24 hour treatment course of high dose NL, which was shown to effectively inhibit BCR-ABL TK activity, it was possible to show that, at least at gene expression level, no significant modulation of the production of GFs highly expressed by CML SPC was present. However a high inter-patient variability was observed which might prevent a firm conclusion from being reached. Moreover as the expression levels were measured at a single time point, it is also possible that early or late changes might have been missed. Finally for those GFs in which protein levels were not measured, it is possible that any modulation at post-translational level would be missed. In

this regard it is worth noting that CSF2RB, whose gene expression levels were not modulated by BCR-ABL kinase inhibition, showed higher modulation when its protein expression was measured following NL treatment. This could be due to the longer NL exposure prior to protein expression analysis causing changes in CSF2RB expression which had been missed by PCR analysis at 24 hours; alternatively an increased turnover/degradation of the receptor following BCR-ABL kinase inhibition might have determined reduced protein levels without any detectable changes at gene expression level. On the other hand, TNF-α was not significantly modulated by BCR-ABL kinase expression at both gene and protein level, suggesting that its production is truly BCR-ABL kinase independent. In that respect it is interesting to note that TNF-α, unlike GM-CSF or IL-3, is one of the GFs also produced in an autocrine fashion by normal $CD34^+$ cells. This would suggest that its autocrine production is not necessarily typical of a cancer cell, although at the same time its higher expression levels in BCR-ABL positive cells support the idea that the levels of expression are influenced by the leukaemic nature of the CML LSC, possibly through other domains of BCR-ABL or genetic/epigenetic changes within the CML LSC which are independent of BCR-ABL protein. It is worth noting that the lack of modulation of autocrine GF production upon BCR-ABL kinase inhibition has also been previously reported for *G-CSF* in BCR-ABL transduced normal $CD34^+$ cells exposed to IM[360]. Similarly the role of other domains of the BCR-ABL oncoprotein, such as the SH2 domain, in autocrine GF production by BCR-ABL positive cells has also been demonstrated[361]. These observations suggest that at least in some instances BCR-ABL TK is either not necessary or not sufficient to determine the aberrant autocrine GF production which has therefore a more complex regulation.

Based on all the above it is possible to conclude that autocrine production of GFs is a more extensive phenomenon in CML than previously appreciated and that it is not always BCR-ABL kinase dependent. On these grounds it is therefore worth investigating it in further detail trying to elucidate, at both molecular and functional levels, the effects that some of these GFs have on CML SPC survival, especially those expressed at higher levels by CML SPC relative to normal HSPC and not modulated by BCR-ABL kinase. In particular it would be interesting to assess if targeting GF production directly, or indirectly through their downstream signalling pathways, could have a therapeutic role in CML SPC eradication. Moreover by assessing the efficacy of combining therapies targeting the autocrine GF production/signalling with a TKI in eradicating CML SPC, the BCR-ABL kinase independence of autocrine GF production/signalling will also be evaluated indirectly. It is reasonable to expect that such combination treatments will yield

additive/synergistic effects only if autocrine GF production is a truly BCR-ABL kinase independent phenomenon.

The role of GM-CSF in normal and leukaemic haemopoieis has already been reviewed in detail (section 1.1.2.1 and 1.3.6.1). Considering also the previously shown autocrine production of IL-3 by CML SPC and the observation of higher levels of expression of their common β chain receptor CSF2RB in CML SPC, it seemed reasonable to focus on the common pathway activated by these ligands/receptors, i.e. JAK2/STAT5. JAK2 could act as a central hub in relaying enhanced prosurvival signals from autocrine and paracrine GFs in CML SPC. This indeed will be the subject of the following chapter of the thesis. It is worth noting that, following the recent clinical development of several small molecule inhibitors of the JAK2 kinase for the management of patients with BCR-ABL negative myeloproliferative disorders, such investigation in CML has become even more relevant as the rapid translation into clinical studies of any promising laboratory results could be possible. The role of TNF-α in normal haemopoiesis has also been extensively reviewed (section 1.1.2.3). However its role in CML biology is much less clear. Given autocrine TNF-α expression levels is higher in CML cells and not modulated via BCR-ABL kinase, its specific role in CML SPC survival and proliferation was also further investigated and will be presented in chapter 5 of the thesis.

4 Results (II) Investigation of the role of JAK2 in the survival and proliferation of CML CD34$^+$ cells

The central role of the intracellular TK JAK2 in normal haemopoiesis and cellular response to several erythropoietic GFs, such as EPO, IL-3 and GM-CSF, has been reviewed in detail in the Introduction (section 1.1.2.1.1). In normal haemopoietic cells JAK2 is activated following GFs binding to their cognate receptors and transduces their signals mainly through activation of its downstream target STAT5. It therefore represents a central hub in relaying signals from several GFs as shown by the profound effects on haemopoieis leading to embryonic lethality of *Jak2* KO mice. Although STAT5 represents the main transcription factor activated by JAK2, the milder phenotype in terms of myeloerythroid development of *Stat5* KO mice compared to *Jak2* KO mice suggests that JAK2 might activate additional signalling pathways besides STAT5 as also shown by recent evidence in other cancer cell models[362].

Constitutive activation of both JAK2 and STAT5 has long been known to be a feature of BCR-ABL positive cells[227,226]. Recent evidence suggests that STAT5 plays an essential role in BCR-ABL induced leukaemogenesis: using *Stat5* KO mouse BM cells transduced with *BCR-ABL* retrovirus, two groups have now demonstrated that *Stat5* is indispensable for the maintenance of BCR-ABL positive leukaemias *in vivo,* although slightly discordant results were obtained regarding the effects of *Stat5* deletion on LSC eradication[236,363]. Furthermore the role of STAT5 as a potential target for leukaemic SPC eradication has been supported by recent reports showing that high levels of STAT5 are protective for BCR-ABL positive cells upon treatment with TKI[364] and that specific targeting of STAT5 activity increases eradication of BCR-ABL positive cells, including primary CML CD34$^+$ cells and CML cells resistant to TKI[365]. Pharmacological interference with STAT5 activation therefore represents an attractive therapeutic strategy in CML. STAT5, though, is a difficult drug target as it is a transcription factor lacking an enzymatic domain that can be targeted and therefore a more simple and logical approach to interfere with STAT5 function would be to inhibit its activating kinase, JAK2. However while in normal haemopoiesis the role of JAK2 in activating STAT5 has been demonstrated, in BCR-ABL positive leukaemias this is a much more controversial area. The relative contribution of JAK2 and BCR-ABL to STAT5 phosphorylation was originally investigated in BCR-ABL positive cell lines by Ilaria and Van Etten. Although BCR-ABL was shown to

phosphorylate JAK2, it also appeared to directly activate STAT5. Moreover a dominant negative JAK2 mutant was unable to prevent the BCR-ABL induced STAT5 activation. This observation led to the conclusion that the role of JAK2 was dispensable for STAT5 activation in BCR-ABL positive cells[230]. More recently Hantschel *et al* have investigated this issue further using *Jak2* KO mouse BM cells transduced with *BCR-ABL* retrovirus and also concluded, based on *in vitro* colony forming assays and *in vivo* transplantation assays, that *Jak2* is dispensable to BCR-ABL induced leukaemias and STAT5 activation[239] and therefore should not be pursued as a therapeutic target in CML.

On the other hand, results of the work presented in the former chapter and previous evidence show that primary CML SPC are capable of autocrine production of GFs, such as GM-CSF and IL-3, which coupled with the normal responses that CML cells show to the same GFs would suggest that activation of STAT5 via JAK2 is also relevant to CML cells survival[270]. As shown in the previous chapter the common β chain receptor for IL-3 and GM-CSF, CSF2RB is also upregulated in CML SPC, suggesting that the GF induced JAK2/STAT5 pathway is active in CML cells and might indeed play a significant role in CML SPC survival, as these cells are likely to respond (possibly even at a higher level compared to normal HSPC) *in vivo* to the same cues produced from the BM stroma. In support of this, *in vitro* experiments have already shown that JAK2 inhibitors can abrogate exogenous GFs antiapoptotic signals in CML $CD34^+$ cells, mainly by reducing the activation of STAT5[260,262]. Alongside its role in transducing GFs signals via STAT5, other roles for JAK2 in BCR-ABL positive leukaemias have also been suggested. Xie *et al* originally showed that JAK2 is necessary for the leukaemogenic potential of BCR-ABL positive cell lines. Using clones stably transfected with kinase inactive JAK2 mutant they were able to show both reduced clonogenic potential and ability to form tumours in nude mice. Interestingly these effects were independent of STAT5 activity[231], but involved downregulation of the transcription factor MYC[240]. More recently the same group has shown in BCR-ABL positive cell lines that JAK2 forms a complex with BCR-ABL necessary for the stabilisation of BCR-ABL and its activity. By disrupting this complex using both JAK2 chemical inhibitors and RNA interference, BCR-ABL kinase stability and activity were dramatically reduced. Targeting JAK2 was effective even in cells harbouring BCR-ABL kinase mutations resistant to standard TKI therapy[366,367]. Neviani *et al* has also shown preliminary data demonstrating a prominent role of JAK2 in maintenance of CML SPC. Interestingly this appears to be secondary to interference of other signalling pathways alongside STAT5. In particular, JAK2 appears to play a prominent role in inactivating the intracellular phosphatase PP2A[368]. PP2A activity is reduced in CML SPC and its

reactivation through JAK2 inhibition can lead to the eradication of CML SPC. Moreover their data also show that JAK2 plays a significant role in the activation of the transcription factor β-catenin, which has also been implicated in the maintenance of LSC[369]. Finally, although in a different disease setting, a recent report has highlighted a role for JAK2 in the cell nucleus as a histone kinase and chromatin modifier, which is again independent from direct STAT5 activation[362].

The recent discoveries of JAK2 activating mutations in BCR-ABL negative myeloproliferative disorders[370,371] has led to the rapid development of numerous JAK2 pharmacological inhibitors, most of which have now reached clinical development[372,373]. As a result, a therapeutic strategy employing JAK2 inhibitors in CML patients could now easily be pursued. It is therefore even more important that a better understanding of the role of JAK2 in BCR-ABL induced leukaemia is obtained before considering a combination of JAK2 and BCR-ABL inhibitors for the treatment of CML patients.

The main aim of the work presented in this chapter was therefore to provide answers to the following key questions on the role of JAK2 in CML using primary $CD34^+$ cells:

1) Does JAK2 play a role for the survival and proliferation of primary $CD34^+$ CML cells, in particular when BCR-ABL kinase is inactive (i.e. in the presence of TKI)?

2) Is JAK2 activation in CML SPC only due to GFs signals? What is the relative contribution of autocrine versus exogenous GFs in the activation of JAK2? Is there a cell autonomous activation of JAK2 in CML SPC via autocrine GFs production or yet unknown signals independent of GFs?

3) Which are the pathways controlled by JAK2 in CML SPC? Has JAK2 an effect at all on STAT5 in CML SPC and/or does it regulate alternative survival pathways?

4) What are the effects of JAK2 inhibition on adult normal $CD34^+$ cells given the known central role of this kinase to normal haemopoiesis?

In a first set of experiments carried out using a non clinically developed JAK2 inhibitor TG, proof of principle of the effects of JAK2 inhibition alone and in combination with TKI in normal and CML $CD34^+$ cells was sought. This included measuring the effects of such a strategy on cell survival, proliferation and STAT5 activation. These experiments were then

followed by similar confirmatory experiments using a clinically licensed JAK2 inhibitor INC now also known as Ruxolitinib. However a more in depth analysis of the role of autocrine and exogenous GFs signals behind JAK2/STAT5 activation and contribution to CML SPC survival was also performed with INC. Finally alternative novel pathways affected by JAK2 inhibition in CML $CD34^+$ cells were investigated using an unbiased proteomic screen of cells treated with INC alone and in combination with TKI.

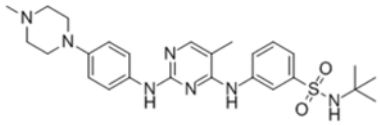
4.1 Investigation of the role of JAK2 in CML and normal CD34$^+$ cells using TG

4.1.1 IC_{50} of TG in CML and normal CD34$^+$ cells

TG is a pyrimidine-based inhibitor targeting JAK2 at nM concentrations (figure 4-1 A). Its IC_{50} for JAK2 in *in vitro* kinase assay has been recorded at 6nM[374]. It is worth noting that, as is often the case with small molecule inhibitors, TG was also able to inhibit other kinases, although at higher IC_{50} concentration (figure 4-1 B). The efficacy of TG in cell lines carrying activating JAK2 mutations has already been reported. In these model systems, TG was able to induce apoptosis and reduce proliferation at a concentration range between 100 and 200nM[374]. Notably IC_{50} in a cell culture system is often higher compared to the value recorded from *in vitro* kinase assays. This reflects the fact that a different readout is used in cell culture systems (apoptosis/viability) which is regulated at different levels and which requires higher inhibitor concentrations to be elicited.

An IC_{50} for TG effects on viability in CML and normal CD34$^+$ cells was measured by our collaborators at the Terry Fox Laboratory -British Columbia Cancer Agency in Vancouver, Canada- Dr Xiaoyan Jiang and Dr Min Chen. In CML CD34$^+$ cells this was recorded at 100nM while in normal CD34$^+$ cells was 250nM (figure 4-1 C). This offered a therapeutic window to work with when trying to assess TG effects on CML and normal CD34$^+$ cells. Based on these preliminary data TG was used at a concentration of 100nM for all following experiments.

A



B

	JAK2	RET	FLT3	JAK3
IC_{50} (nM)	6	17	25	169

C

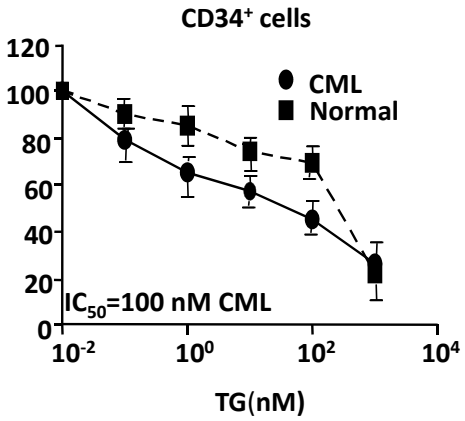


Figure 4-1 Molecular structure of TG and its IC_{50} in *in vitro* kinase assays and primary CML and normal $CD34^+$ cells
Primary CML and normal $CD34^+$ cells were cultured in SFM+HiGF (from which SCF had been removed) in the presence of increasing concentrations of TG. Viability was assessed by trypan blue dye exclusion method after 72 hours and expressed as % of UT (courtesy of Dr X. Jiang and M. Chen).

4.1.2 Assessment of the effects of TG+TKI combination on CML and normal CD34$^+$ cells viability and apoptosis induction

In order to assess the effects of combining JAK2 inhibition with BCR-ABL kinase inhibition, CML and normal CD34$^+$ cells were treated with TG 100nM, DA 150nM, IM 5μM, NL 5μM and their combination. The concentrations of TKI were chosen to obtain maximal BCR-ABL kinase inhibition while being within (DA and IM)[329,375], or slightly above (NL)[359], the peak plasma concentrations achievable in patients. It has to be noted that in these experiments, cells were cultured in SFM+HiGF from which SCF had been removed to exclude any effects of IM secondary to inhibition of the SCF receptor TK, c-KIT. IM is known to inhibit c-KIT at nM concentration[283].

The percentage of viable cells following 72 hours in culture in the presence of each inhibitor was reduced by 50 to 60% compared to UT CML CD34$^+$ cells. Moreover a further 50% reduction was observed in all combination arms compared to single agent TKI alone, with the difference reaching statistical significance for the IM versus IM+TG comparison ($p=0.02$) (figure 4-2 A left panel). When the actual number of viable cells was measured over 3 days, a significant 50% reduction in the expansion of CML CD34$^+$ cells was observed at 72 hours when comparing all 3 TKI arms as a group with the 3 combination arms ($p<0.001$) (figure 4-2 A right panel), but it should be noted that the number of cells following treatment was still higher than input cell number. Although a degree of reduction in viability was also seen in normal CD34$^+$ cells, mainly following combination treatment, this was not of the same magnitude and not statistically significant (figure 4-2 B left panel). Similarly no obvious difference was observed in the expansion of normal CD34$^+$ cells following treatment (figure 4-2 B right panel).

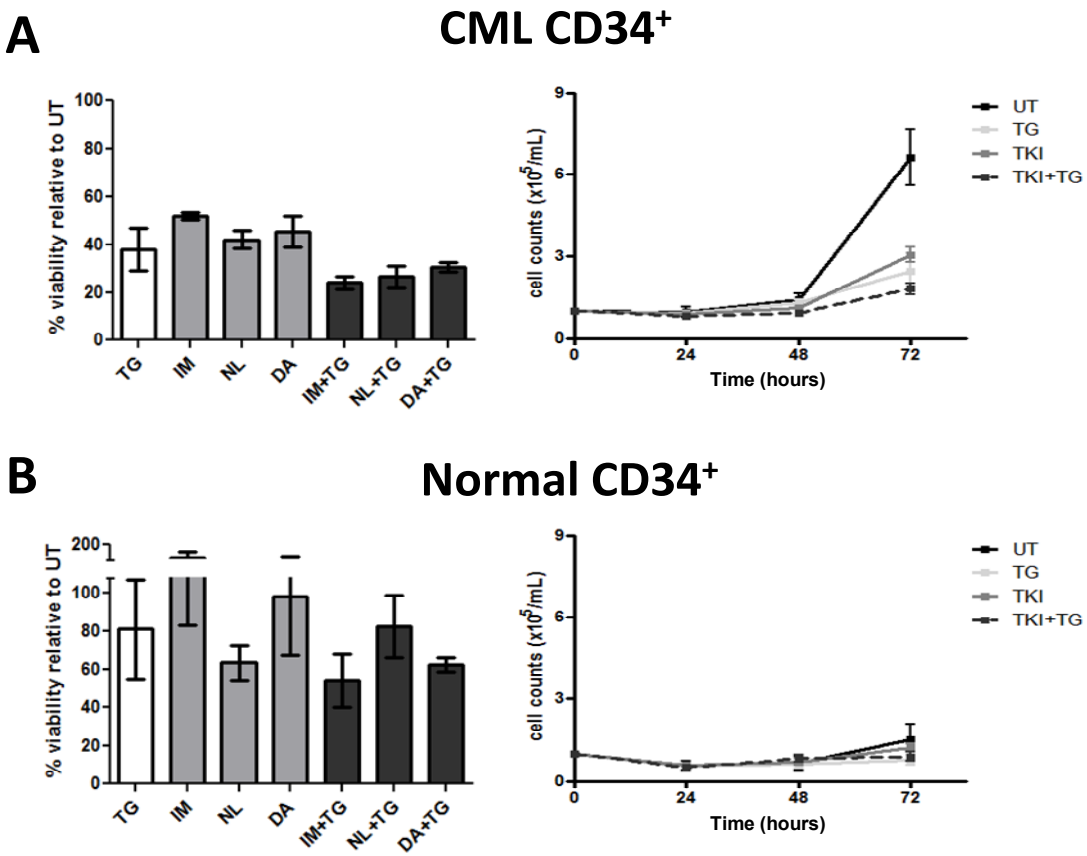


Figure 4-2 Effects of the combination of TG+TKI on CML and normal CD34$^+$ cell viability and cell expansion
Viability was assessed in 3 CML and normal CD34$^+$ cell samples by trypan blue dye exclusion method at 72 hours following treatment (left panels). Cell expansion was recorded in the same samples by counting number of viable cells in each culture at 24, 48 and 72 hours post treatment. Results for the 3 TKI and for the 3 combination arms were grouped together in the right hand panels.

In order to confirm the above finding by an alternative method, annexin-V staining was used to measure apoptosis following 72 hours culture with each drug and their combination. Again all the combination arms produced significantly higher levels of apoptosis compared to single agent treatment in CML CD34$^+$ cells (2-3 fold increase for each arm, $p<0.05$ for all comparisons), while no significant increase in apoptosis level was noted with TG in combination with TKI in normal CD34$^+$ cells (figure 4-3 A and B). Given the consistent results obtained for the 3 TKI, their results were combined for all the subsequent analyses in order to assess a TKI drug class effect versus a TG+TKI effect.

The phenotype of viable (annexin-V and 7AAD double negative) CML SPC following 72 hours drug exposure was also assessed using CD34 and CD38 staining by flow cytometry. The percentage of cells within each population (CD34$^+$ CD38$^+$, CD34$^+$ CD38^{low} and CD34$^+$ CD38$^-$) was multiplied by the total viable cell count at the same time point to give the total number of viable cells for each population following culture. This was then compared to the input number of cells within each subpopulation (baseline). In the single

agent TG and TKI treatment arms, a reduction in the number of more mature $CD34^+$ $CD38^+$ progenitor cells below baseline number of cells was observed, but more primitive $CD34^+$ $CD38^{low}$ cells (a population with greater short-term expansion potential) and $CD34^+$ $CD38^-$ cells (a stem cell-enriched population) still expanded compared to baseline, although less than in the UT arm (2 and 3-fold fewer cells in cultures with TKIs or TG, respectively, as compared to untreated controls after 3 days in culture). This was consistent with reported observations that more primitive CML cells are less sensitive to TKI treatment than the more mature cells[328,337]. However, in the TKI+TG treatment arm, a further reduction in the numbers of the $CD34^+$ $CD38^{low}$ cells compared to either of the single agents alone and, more strikingly, an absolute reduction in the number of $CD34^+$ $CD38^-$ cells (>10-fold reduction) was noted (figure 4-3 C).

Overall these data show that the combination of TG+TKI was more effective in eradicating CML $CD34^+$ cells compared to single agent TKI treatment. Moreover phenotypic analysis of viable cells suggests that there was also a more potent effect on the most primitive stem cell compartment with the combination arm. Only mild toxic effects were seen on normal $CD34^+$ cells in this short term liquid culture assay.

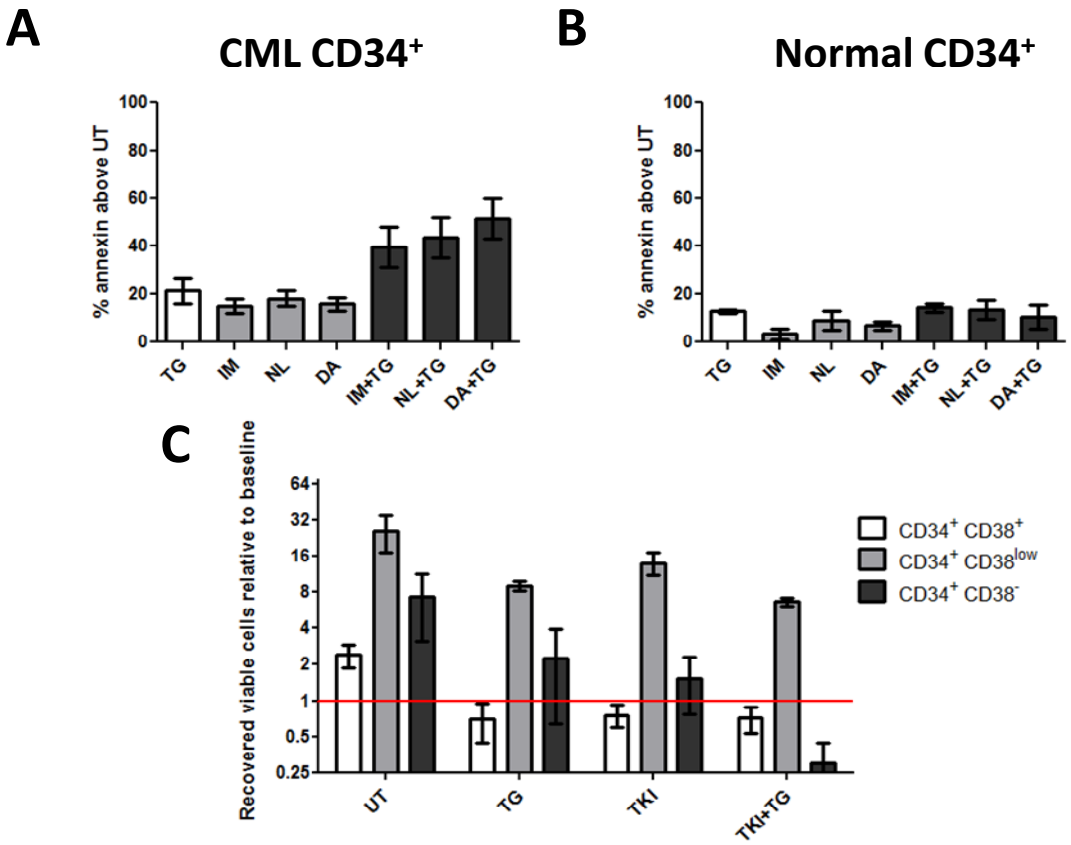


Figure 4-3 Apoptosis induction in CML and normal CD34$^+$ cells and phenotype of remaining viable CML cells following exposure to TG+TKI

Apoptosis was measured by annexin-V/7AAD staining in 3 CML and normal CD34$^+$ cell samples cultured with either single agent drugs or their combination at 72 hours following treatment (A and B). CD34$^+$ CML cells (n=3) were cultured for 72 hours in the presence of IM, NL, DA or TG alone, or a combination of TKI with TG. The phenotypes of viable (annexin-V/7-AAD double negative) cells were then determined by flow cytometry based on their expression of CD34 and CD38. The total number of viable cells within each subpopulation was then calculated based on viable cell counts at 72 hours and compared to the starting number of cells within each subpopulation (baseline), represented by the red line (C).

4.1.3 Assessment of the effects of TG+TKI combination on CFC output in CML and normal CD34$^+$ cells

In order to measure the effects of combining TG with TKI on CFC, CML and normal CD34$^+$ cells were cultured in the presence of TG and TKI for 6 days. At day 3 cells were washed in PBS, resuspended in fresh media and drugs were added again to the cultures. At both day 3 and day 6 following drug washout, 3,000 cells were taken from each treatment arm and resuspended in 300μL of fresh medium and then added to 3mL of Methocult H4034 for CFC assay as described in 2.3.1.7.

At day 3 both TKI and TG+TKI CML CD34$^+$ treated cells showed a reduction in CFC output compared to CFC output from the same sample at day 0 (baseline); as expected the reduction in CFC became even bigger following a further 3 days of treatment to day 6. At both time points however the combination arm produced a more marked reduction in CFC output compared to TKI treated CML cells with a relative reduction of 35% in CFC output at day 3 and 84% at day 6 ($p=0.03$ at day 6) and almost complete elimination of CFC in absolute numbers by day 6 with the combination treatment (figure 4-4 A). Normal CD34$^+$ cells treated with TKI predictably showed no change in CFC at day 3 and day 6 compared to baseline. The combination arm showed signs of early toxicity as it caused a relative reduction of 43% in CFC output in comparison to TKI treated cells at day 3 ($p=0.012$). However following another 3 days in culture no further reduction in CFC output was seen for TG+TKI treated cells (relative CFC reduction compared to TKI of 32%, $p=0.019$) (figure 4-4 B).

Overall these data show that the combination of TG+TKI was more effective than TKI treatment in eradicating CML CFC and the effect increased over time. While a degree of early toxicity was observed with the combination treatment on normal CFC at day 3, this did not increase over time thus suggesting that the carefully selected concentration of TG used in this study provides a therapeutic window for the combination arm.

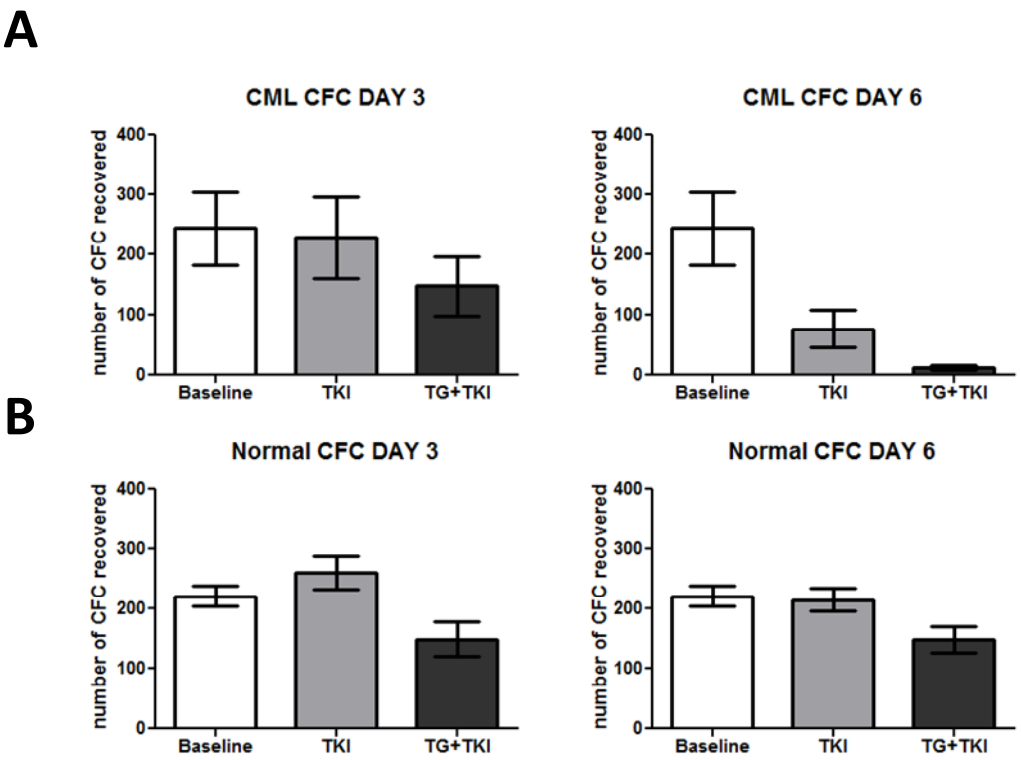


Figure 4-4 CFC output of CML and normal CD34$^+$ cells following treatment with TG+TKI
3,000 cells from 2 CML and 3 normal CD34$^+$ samples were plated in standard CFC assays after 3 (left) or 6 (right) days of prior incubation in suspension cultures with TKI or TG+TKI. Number of colonies recovered from each arm is plotted together with the number of colonies recovered at day 0 from 3,000 CD34$^+$ cells (baseline). The number of colonies following 3 and 6 days in culture was also adjusted for the expansion *in vitro* of CD34$^+$ cells in each arm relative to baseline.

4.1.4 Assessment of the effects of TG+TKI combination on STAT5 phosphorylation levels in CML and normal CD34$^+$ cells

In order to clarify the mechanism behind the effects seen with the combination therapy, levels of p-STAT5 were measured by intracellular flow cytometry in CML CD34$^+$ cells at various time points. P-STAT5 is a known surrogate marker for BCR-ABL kinase activity, although it is also activated by JAK2.

Figure 4-5 panel A shows that p-STAT5 levels were significantly reduced by the combination treatment compared to either single agent alone, at both 24 and 72 hours following treatment (TKI versus TG+TKI comparison - p-STAT5 19% absolute reduction at 24 hours, $p<0.001$ and 26% absolute reduction at 72 hours, $p<0.001$). Overall these results show that the combination of TG+TKI enhances the inhibition of BCR-ABL and JAK2 kinase activity in CML SPC in a durable fashion. Levels of p-STAT5 were also measured in equivalent cultures of normal CD34$^+$ cells. As expected normal CD34$^+$ cells expressed lower levels of p-STAT5 when compared to CML CD34$^+$ cells (35% reduction in p-STAT5 levels between normal and CML, $p=0.09$) (figure 4-5 B). Moreover phosphorylation levels were not significantly changed following treatment with TG or TKI in normal CD34$^+$ cells at 24 hours (figure 4-5 C).

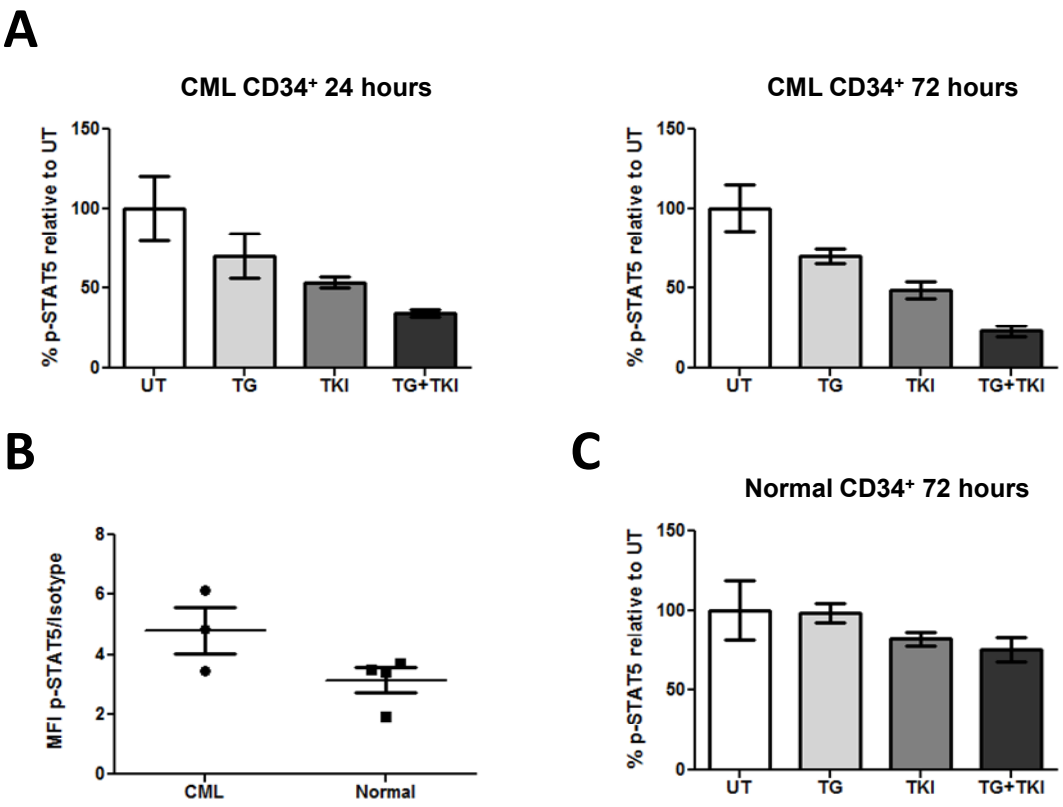


Figure 4-5 Comparison of p-STAT5 levels in CML and normal CD34$^+$ cells following treatment with TG+TKI

p-STAT5 (A) levels were measured by intracellular flow cytometry in 3 CML CD34$^+$ samples after 24 (left panels) or 72 (right panels) hours of incubation in suspension cultures with TG, TKI or TG+TKI. p-STAT levels were measured in 3 CML and 4 normal CD34$^+$ samples after overnight recovery in SFM+HiGF (B) and in normal CD34$^+$ cells (n=3) following 24 hours of incubation in suspension cultures with TG, TKI or TG+TKI (C). Levels of p-STAT5 were calculated based on MFI of antibody stained samples relative to isotype stained samples. For changes following treatment, the average of UT values was normalised to 100% and levels in treatment arms expressed as % change from UT.

4.1.5 Assessment of the effects of TG+TKI combination on *in vivo* engraftment of CML CD34$^+$ cells

To assess the effects of combined TG plus IM treatment on *in vivo* leukaemogenic activity of primary CML SPC, transplantation assays of CD34$^+$ CML cells into non obese diabetic/severe combined immunodeficient (NOD/SCID) interleukin 2 receptor γ-chain-deficient (NSG) mice were performed by our collaborators in Vancouver. 6x10^6 CML CD34$^+$ cells per treatment condition from three CML patients were exposed to 1μM IM, 100nM TG, or their combination for 72 hours. Following the 3 day drug exposure in culture, all recovered cells were washed and injected intravenously into 8-10-week old, sublethally irradiated (350 cGy) NSG mice (three mice/per group for each patient sample). At 16 weeks, the levels of both human CD45$^+$ and CD34$^+$ leukaemic cells regenerated in the BM of transplanted NSG mice following IM+TG treatment of primary CD34$^+$ CML cells *in vitro* was reduced compared to cells pretreated with IM or TG alone (figure 4-6 A and B). In particular CD34$^+$ cells became almost undetectable in the BM of mice injected with cells pretreated *in vitro* with the TG plus IM combination (0.13% CD34$^+$ cells only). Over 90% of the human cells obtained from mice transplanted with CML cells not exposed to drug were *BCR-ABL* positive by FISH (due to low cell numbers FISH was not possible in the other arms) (figure 4-6 C). However qRT-PCR at 4 weeks posttransplant confirmed that *BCR-ABL* transcript levels in FACS purified CD45$^+$ BM cells were reduced significantly in cells pretreated with combination treatment compared to cells pretreated with either single agent alone ($p<0.001$ for both TKI versus TG+IM and TG versus TG+IM comparison).

Overall these results show that IM+TG combination reduces the leukaemogenic activity of CML LSCs more effectively than IM or TG alone.

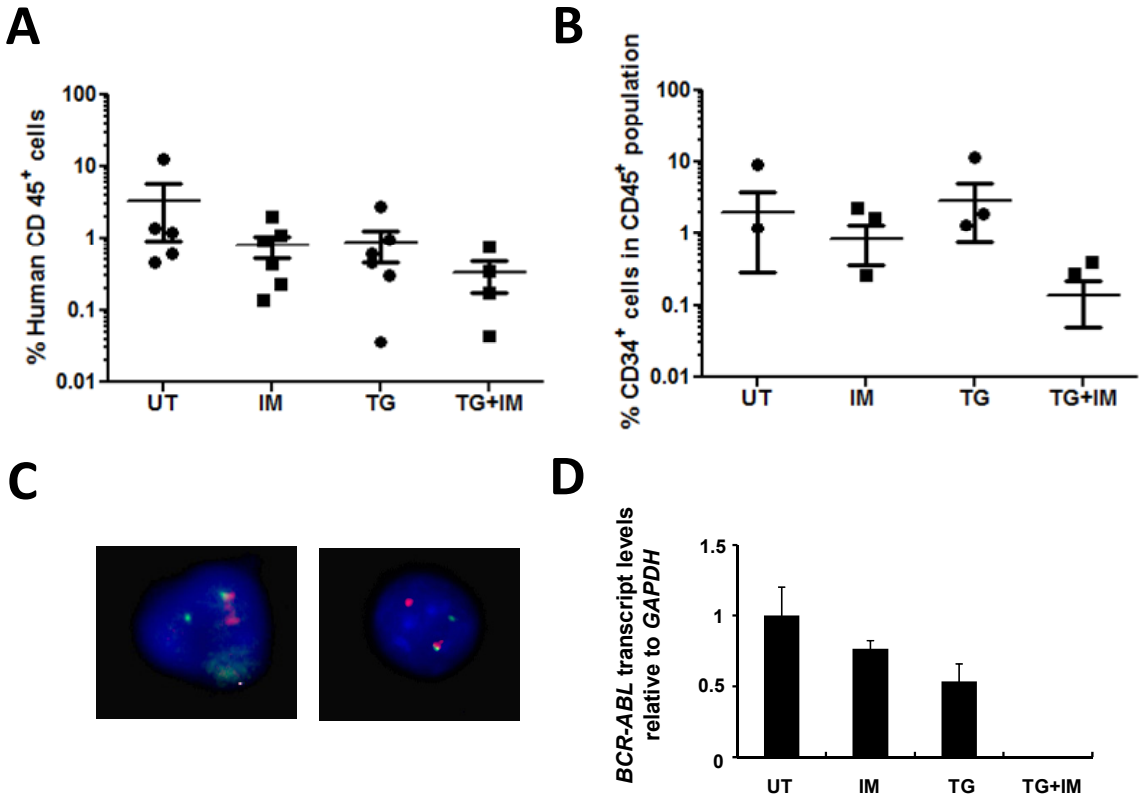


Figure 4-6 Engraftment of CML CD34$^+$ cells in immunodeficient mice following TG+TKI treatment

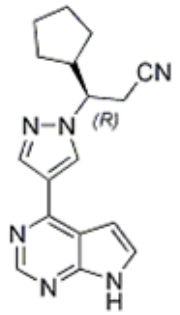
CML CD34$^+$ cells (n=3) were cultured for 72 hours with IM, TG or IM+TG or left UT. Following this they were washed and injected intravenously into NSG mice. After 16 weeks, BM aspirates were obtained and the presence of human CD45$^+$ (A) and CD34$^+$ (B) cells measured by flow cytometry. Representative FISH pictures of human *BCR-ABL* positive cells present in the BM of mice transplanted with UT CD34$^+$ CML cells (C). *BCR-ABL* transcript levels at 4 weeks in FACS purified CD45$^+$ BM cells from mice transplanted with CML CD34$^+$ cells either UT or pretreated with IM, TG or their combination. Transcript levels were expressed relative to *GAPDH* (housekeeping gene) transcript levels (D). Animal experiments were performed in the Animal Resource Centre of the British Columbia Cancer Agency Research Centre, using procedures approved by the Animal Care Committee of the University of British Columbia (Vancouver, Canada). Figures are courtesy of Dr Xiaoyan Jiang, Min Chen and Kyi Min Saw.

4.2 Investigation of the role of JAK2 in CML and normal CD34$^+$ cells using the clinically developed JAK2 inhibitor INC

4.2.1 Assessment of IC_{50} for INC in CML CD34$^+$ cells

INC is a potent, selective and orally bioavailable JAK1/2 inhibitor. In kinase assays it was shown to inhibit the above kinases at low nM concentrations, while also having effects on other kinases at higher concentrations (figure 4-7 A and B). However when the activity of INC was measured in a more physiological setting using GFs stimulated whole blood assay, the IC_{50} was observed to be between 200 and 300nM for both JAK1 and JAK2, which is likely to be a more genuine reflection of the IC_{50} in cellular assays and *in vivo*. In preclinical studies INC has shown efficacy in reducing survival and proliferation of JAK2 mutant cell lines and primary MNC from patients with myeloproliferative disorders at concentrations ranging between 100 to 200nM. Moreover it has also shown efficacy in an *in vivo* model of myeloproliferative disorder[376]. On the basis of these promising results, INC underwent a rapid clinical development which has led to its licensing for the treatment of primary myelofibrosis following encouraging results from phase 3 clinical trials[372,373]. Clinical studies with this compound are currently ongoing in other myeloproliferative disorders and both haematological and solid tumours.

A



B

ENZYME	IC_{50} (nM)
JAK1	3.3
JAK2	2.8
TYK2	19
JAK3	428

Figure 4-7 Molecular structure of INC (A) and its IC_{50} towards JAK1/2/3 and TYK2 in *in vitro* kinase assays (B)

INC efficacy in CML CD34$^+$ cells was assessed in preliminary experiments by measuring both viable cell numbers and induction of apoptosis at 72 hours as a readout. In a first set of experiments a wide range of concentrations from 1 to 1,000nM (which is the maximal concentration (C_{max}) achieved in patient plasma[377]) was tested. A concentration of INC between 100 and 1,000nM appeared to reduce by about 50% the viable cell number (figure

4-8 A left panel). Moreover an increase in apoptosis induction was also seen starting from 100nM (figure 4-8 A right panel). A further titration experiment was carried out to identify more precisely the IC_{50} for INC using a narrower range of concentrations from 200 to 1000nM. Viable cell counts were reduced by 50% starting at 200nM with no further reduction with increasing concentrations. Again a degree of induction of apoptosis was also observed starting at 200nM which did not increase significantly with higher concentrations (figure 4-8 B). On the basis of these experiments a concentration of 200nM was chosen for all subsequent experiments, in line with the one used in published preclinical studies in JAK2 mutated cell lines.

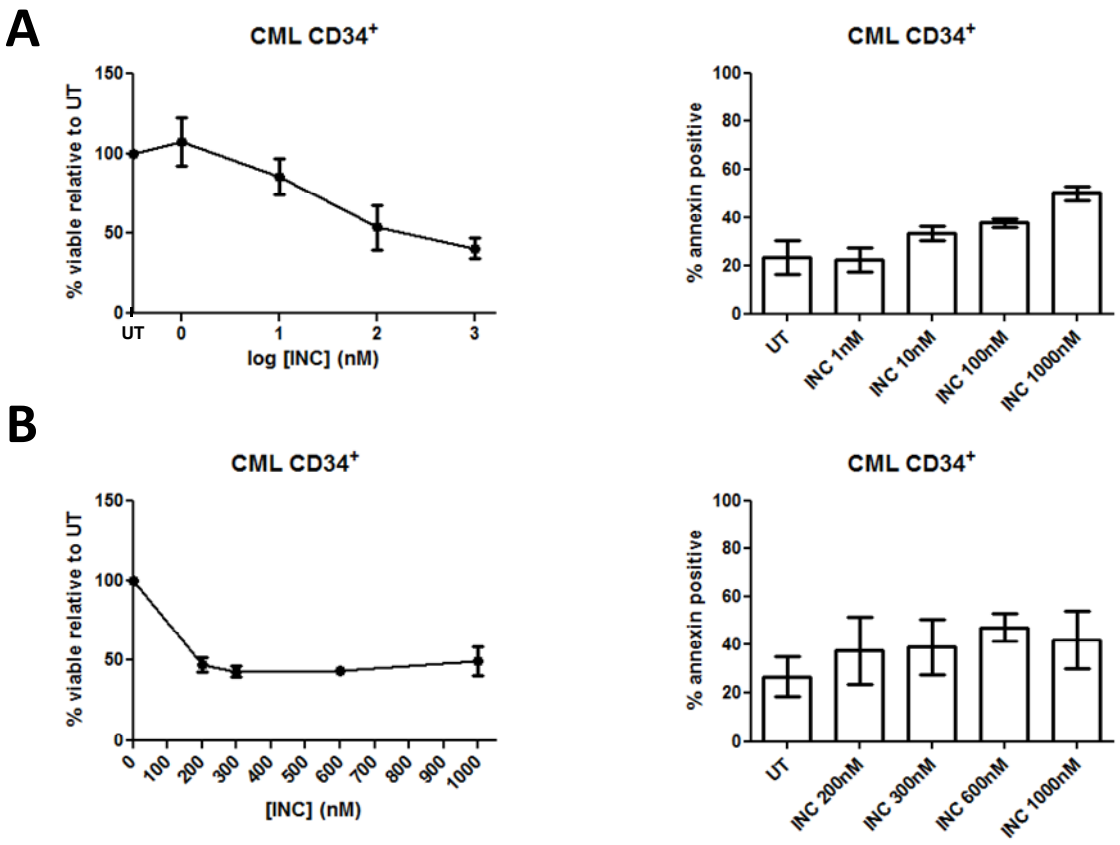


Figure 4-8 Assessment of IC_{50} for INC in CML $CD34^+$ cells
CML $CD34^+$ cells (n=3) were cultured in SFM+PGF in increasing concentrations of INC for 72 hours at which point viable cell count was measured using trypan blue exclusion method (A and B left panels) and apoptosis measured by annexin-V/7AAD staining (A and B right panels).

4.2.2 Assessment of the effects of NL+INC combination in inducing apoptosis and on CFC output in CML CD34$^+$ cells

Having defined an effective concentration of INC in CML CD34$^+$ cells, further experiments focused on the effects of combining INC with TKI to assess the efficacy of this combination treatment. It has to be noted that all experiments were carried out in SFM+PGF (unless otherwise stated) in order to mimic a more physiological condition for CML cells based on the work produced by Bhatia *et al*[275]. NL was chosen as a TKI based on its higher potency and specificity and was used at a concentration of 5μM to achieve maximal BCR-ABL kinase inhibition as previously explained (see introduction of chapter 3). The effects of combining NL+INC on inducing apoptosis and on CFC output in CML CD34$^+$ cells were first assessed.

As shown in figure 4-9 panel A, the combination of NL+INC was able to induce higher levels of apoptosis compared to either single agent alone, at both 48 and 72 hours ($p<0.05$). After 72 hours culture in the presence of the same drug, 3,000 cells were recovered for CFC assay. The colonies were also characterised morphologically in burst forming unit – erythroid (BFU-E), colony forming unit – erythroid (CFU-E) and colony forming unit – granulocyte and macrophage (CFU-GM). The former two represent erythroid colonies deriving from more mature progenitors compared to CFU-GM. NL alone was able to reduce erythroid colonies (BFU-E and CFU-E), but not CFU-GM, compared to baseline, while the combination treatment caused a more marked reduction in erythroid colonies and also a non significant reduction in the number of CFU-GM compared to baseline. When the total CFC output was considered, a significant reduction with NL+INC compared to NL was observed (50% relative reduction, $p=0.02$) (figure 4-9 B). It was also noticeable that the morphology of the colonies changed following combination treatment with mainly small colonies of 100 to 200 cells left (figure 4-9 C).

Overall these data show that the combination of NL and INC was more effective than NL treatment alone in eradicating CML CD34$^+$ cells, including CFC, in short term culture assays.

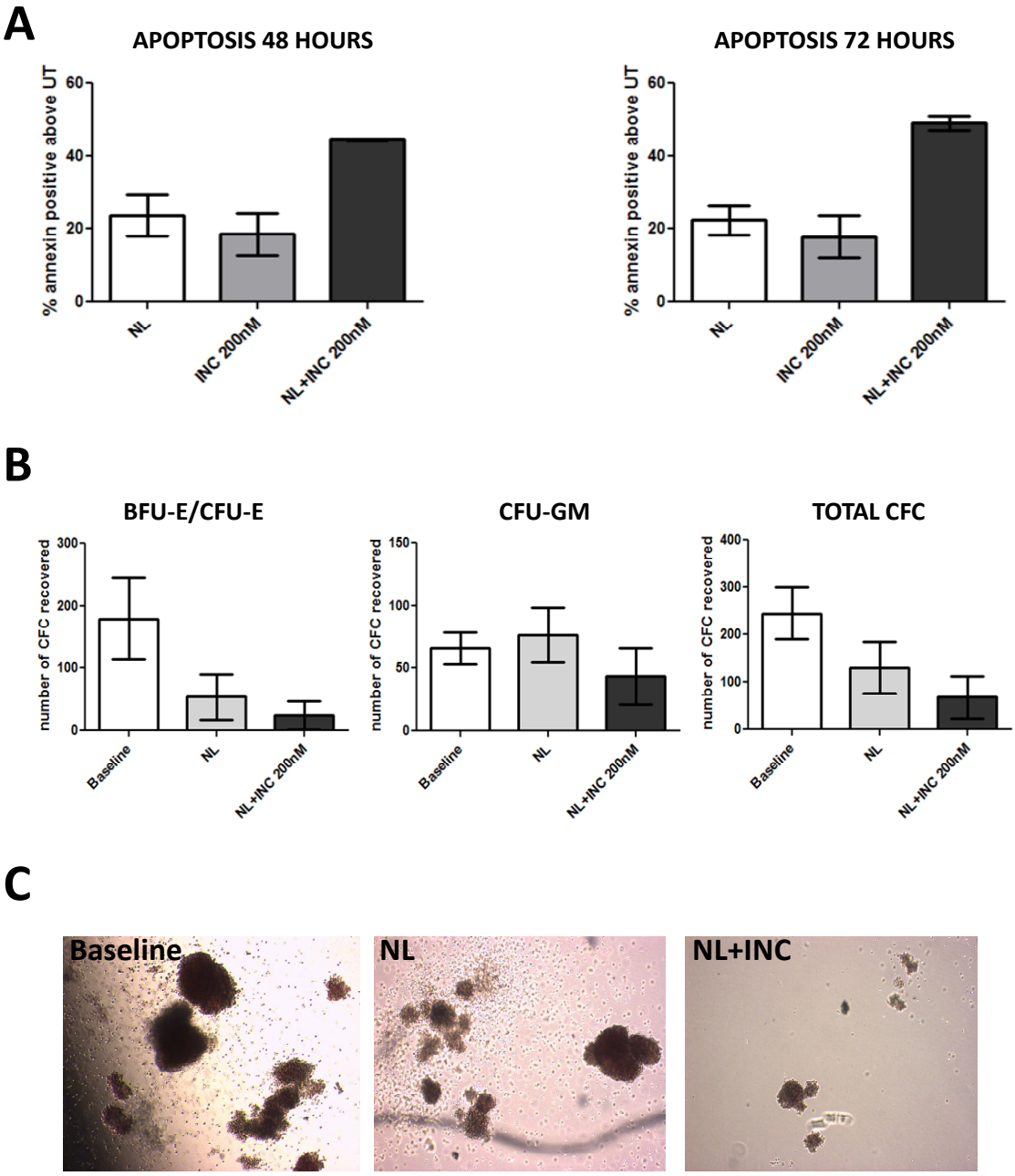


Figure 4-9 Apoptosis induction and CFC output in CML CD34$^+$ cells following treatment with NL+INC

CML CD34$^+$ cells (n=3) were cultured in SFM+PGF with NL, INC, their combination or left UT. At 48 and 72 hours apoptosis levels were measured by annexin-V/7AAD staining (A). At 72 hours, 3,000 cells from each of the arms were plated in standard CFC. Number of colonies recovered in total and based on their morphology from each arm and at baseline is plotted in panel B. The number of colonies following 72 hours culture was adjusted for the expansion of CD34$^+$ cells *in vitro* in each arm relative to baseline. Representative picture of the size and morphology of recovered CFC in each treatment arm is shown in panel C.

4.2.3 Assessment of the effects of NL+INC combination on both proliferating and non proliferating CML CD34$^+$ cells

In order to assess the effects of the NL+INC combination on both proliferative and quiescent CML CD34$^+$ cells, CFSE staining was used. Using this technique, research carried out in our laboratory has previously shown that all TKI are unable to reduce the CFSE^{max} population which is enriched with most primitive, quiescent cells[328-330]. This observation has led to the conclusion that the main effect of TKI on CML SPC is antiproliferative rather than apoptotic and is currently considered to be one of the main reasons behind the persistence of CML stem cells even in patients who have achieved deep molecular responses to TKI. It was therefore interesting to see if the effects induced on bulk CD34$^+$ cells and CFC by the NL+INC combination resulted in a reduction of the CFSE^{max} population.

Figure 4-10 (panel A) shows that while NL and INC as single agents started to reduce the recovered cells in the second division (recovered cells in division 2 for both NL and INC versus UT, $p<0.001$), the combination arm had a statistically significant effect on cells recovered from the first division (recovered cells in division 1 for NL+INC versus UT, $p<0.001$). Moreover while NL even at high concentrations of 5μM had no effect on the undivided recovered cells, both INC and even more the NL+INC combination showed a bigger reduction of cells recovered in the undivided population gate, although the differences did not reach statistical significance. Moreover when the total recovery of CD34$^+$ cells was measured a further decrease was observed with the combination treatment, which became significant when compared to UT ($p<0.05$) (figure 4-10 B).

In order to measure the effects on the CFSE^{max} population using a different approach, CML CD34$^+$ cells were co-stained with CFSE and annexin-V and the levels of apoptosis within the CFSE^{max} population measured at 72 hours. Interestingly the NL+INC combination produced higher levels of apoptosis within the CFSE^{max} population, with the difference being significant when compared to both UT and NL treated cells (respectively $p<0.01$ and $p<0.05$) (figure 4-10 C).

Overall these data show that NL+INC together have a more profound effect on the quiescent stem cell compartment compared to either agent alone.

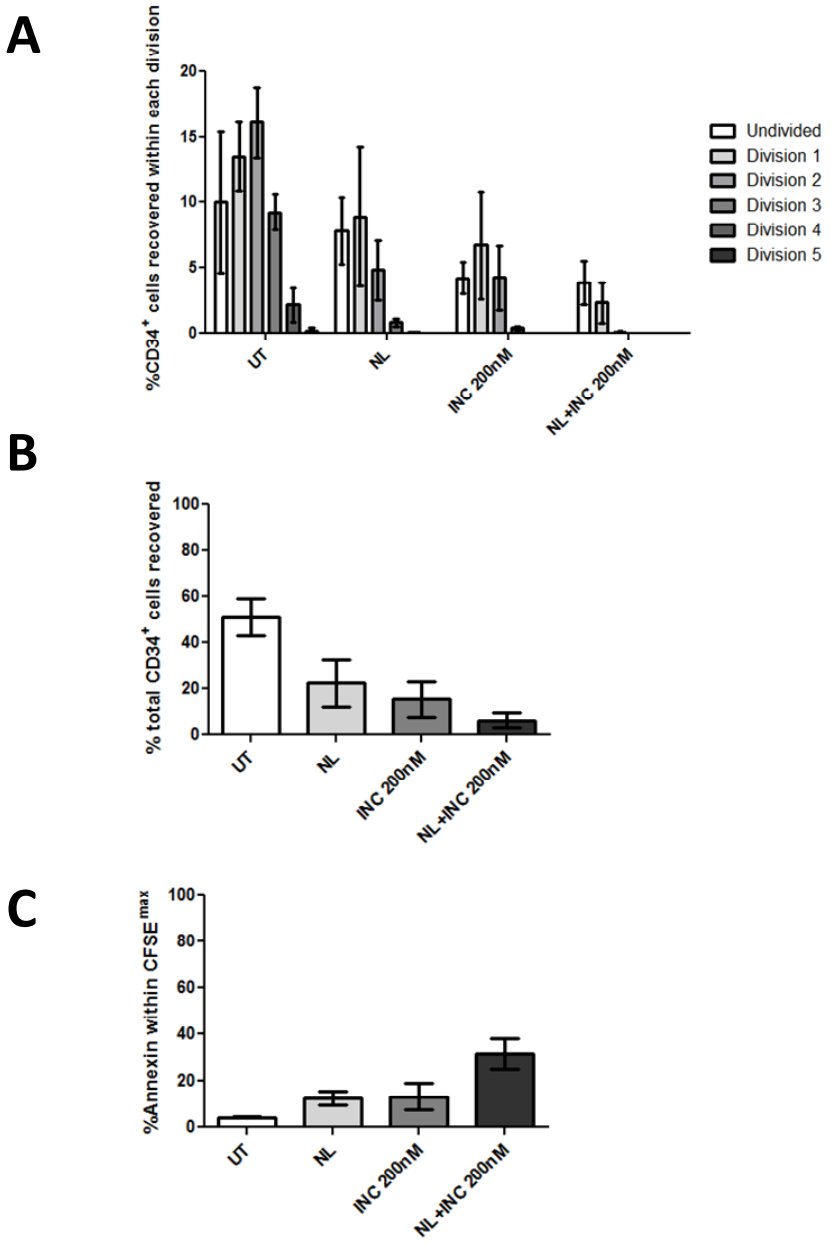


Figure 4-10 Effects of NL+INC on quiescent and proliferative CML CD34$^+$ cells
CML CD34$^+$ cells (n=3) were stained with CFSE and then cultured for 72 hours with NL, INC, their combination or left UT. At 72 hours cells from each arm were co-stained with annexin-V and flow cytometry analysis performed. Percentages of starting CD34$^+$ cells recovered within each division (A) and in total (B) following treatment were calculated as explained in 2.3.2.3. Percentage apoptosis within CFSE^{max} was measured by gating on the population double positive for maximal CFSE expression and annexin-V staining.

4.2.4 Assessment of the effects of INC alone and in combination with NL on normal CD34$^+$ cells

Having shown an increased efficacy for the NL+INC combination on CML CD34$^+$ cells, the toxicity of this treatment to normal CD34$^+$ cells was also assessed. It is important to note that in these experiments normal CD34$^+$ cells had to be cultured in SFM+HiGF as they will quickly undergo apoptosis within 24 hours *in vitro* culture in low GFs concentrations, such as those present in SFM+PGF.

Although an increased apoptosis of normal CD34$^+$ cells at both 48 and 72 hours was seen with combination treatment when compared to either of the single agents alone (not significant at 48 hours and $p<0.05$ at 72 hours for the NL+INC versus NL comparison), the magnitude of this effect was reduced compared to that seen with the combination treatment on CML CD34$^+$ cells, whereby NL+INC caused about 50% more apoptosis relative to UT and about 25% more apoptosis relative to NL (48 and 72 hours; figure 4-9). In normal CD34$^+$ cells the corresponding figures were halved with NL+INC causing about 20% more apoptosis relative to UT and 12% more apoptosis relative to NL at both time points (figure 4-11 A). CFC output was also measured following 3 days drug treatment and in this case no major difference was observed in the effects caused by NL+INC on normal and CML CD34$^+$ cells. In fact NL+INC caused a 65% reduction relative to baseline ($p=0.03$) in total CFC output in normal CD34$^+$ (figure 4-11 A right panel) versus a 73% reduction in total CFC output in CML CD34$^+$ cells (figure 4-9). CFSE staining was then used to assess the effects of the NL+INC combination on both proliferating and quiescent normal CD34$^+$ cells to define further the toxic effects of this treatment. As expected compared to UT, both NL and INC as single agents had an antiproliferative effect as demonstrated by the increased recovery of CD34$^+$ cells in the early divisions, including the CFSE^{max} population (figure 4-11 B). Moreover an overall increase in the total CD34$^+$ cells recovered was also observed with NL or INC, which is likely due to reduced differentiation of CD34$^+$ cells because of their antiproliferative effects (figure 4-11 C left panel). NL+INC had an even more marked antiproliferative effect on the cells within the early divisions causing an increase in CD34$^+$ cells recovered in CFSE^{max} gate and division 1. Overall CD34$^+$ cells recovery was only slightly reduced compared to UT after NL+INC treatment (figure 4-11 C left panel). Finally levels of apoptosis within the CFSE^{max} population were not significantly increased with NL+INC combination (figure 4-11 C right panel).

Overall these data show that NL+INC combination causes a degree of toxicity on more mature normal progenitors as shown by the increased apoptosis in bulk $CD34^+$ cells, reduced CFC output and reduced total $CD34^+$ cells recovery. However this effect is not of the same magnitude as the one seen in CML $CD34^+$ cells. Moreover NL+INC combination has mainly an antiproliferative effect on the most primitive and quiescent normal stem cell pool (which is represented by the $CFSE^{max}$ population). These effects are different from those observed in CML $CD34^+$ cells where a reduction in the quiescent population was seen with combination treatment, together with induction of apoptosis within the $CFSE^{max}$. These latter observations would suggest that this combination might preferentially target the most primitive CML LSCs, but spare the corresponding normal counterparts.

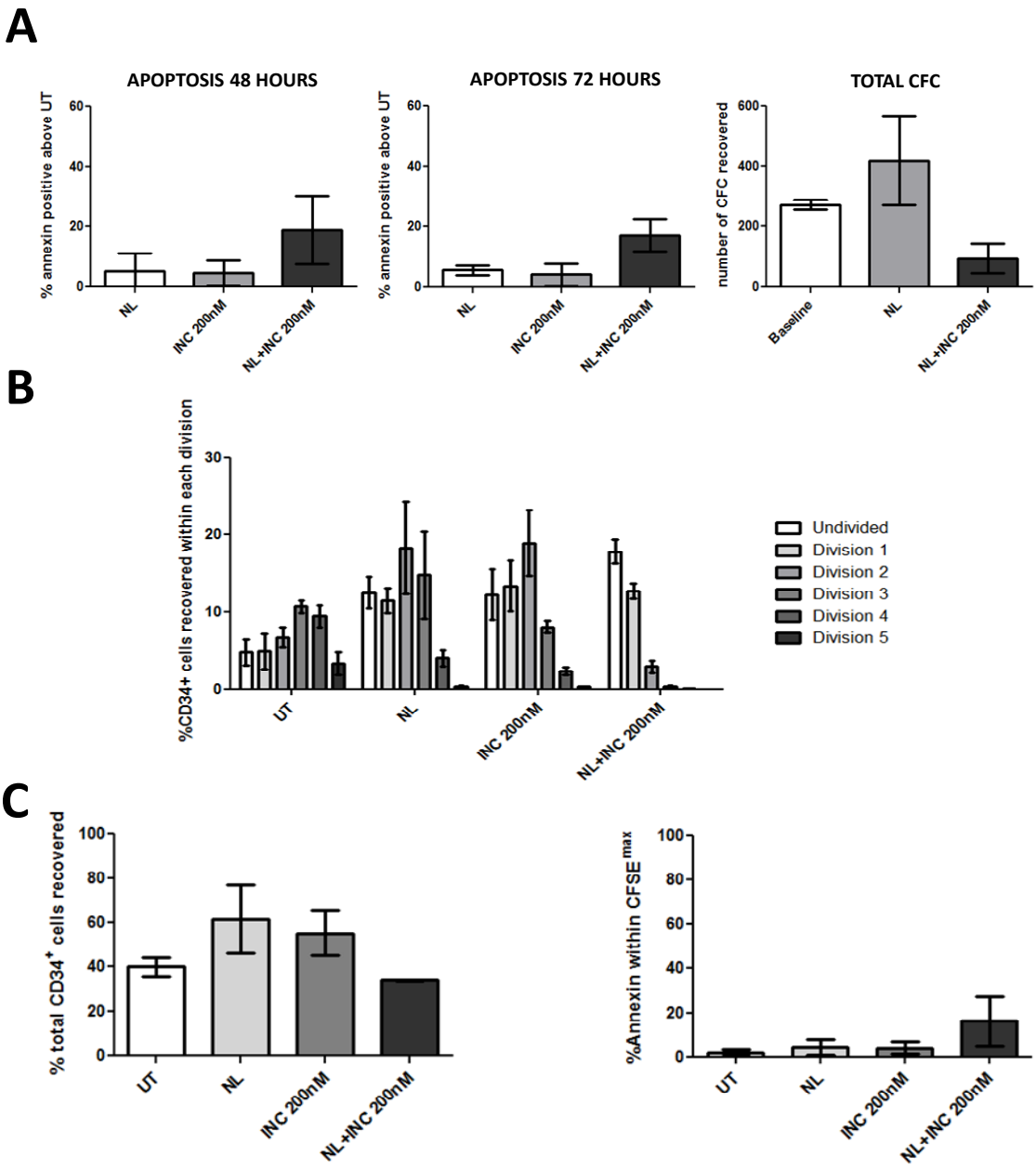


Figure 4-11 Effects of NL+INC combination on normal CD34$^+$ cells
Normal CD34$^+$ cells (n=3) were cultured with NL, INC, their combination or left UT in SFM+HiGF. Apoptosis was measured at 48 and 72 hours by annexin-V staining (A left and central panel). At 72 hours, 3,000 cells from each treatment arm were plated in standard CFC assays (A right panel). The number of colonies following 72 hours culture was also adjusted for the expansion of CD34$^+$ cells *in vitro* in each arm relative to baseline. CFSE stained cells (n=3) were analysed by flow cytometry after 72 hours culture in the same conditions. Percentages of starting CD34$^+$ cells recovered within each division (B) and in total (C left panel) following treatment were calculated as explained in 2.3.2.3. Percentage apoptosis within CFSE^{max} was also measured (C right panel) as described in figure 4-10.

4.2.5 Assessment of the effects of NL+INC combination on STAT5 and JAK2 phosphorylation levels in CML CD34$^+$ cells

In order to clarify the mechanism behind the increased efficacy of the NL+INC, changes in phosphorylation of STAT5 and JAK2 in CML CD34$^+$ cells following treatment with either agent alone or their combination were measured using intracellular flow cytometry.

NL reduced levels of p-STAT5 and p-JAK2 thus suggesting that they are both under control of BCR-ABL kinase. However the addition of INC caused a further 30% and 40% relative reduction respectively in p-STAT5 and p-JAK2 levels compared to NL ($p<0.05$ for both p-STAT5 and p-JAK2) (figure 4-12).

Overall these data show that the combination of NL+INC has more profound inhibitory effects on the signalling through JAK2 and STAT5 than either agent alone.

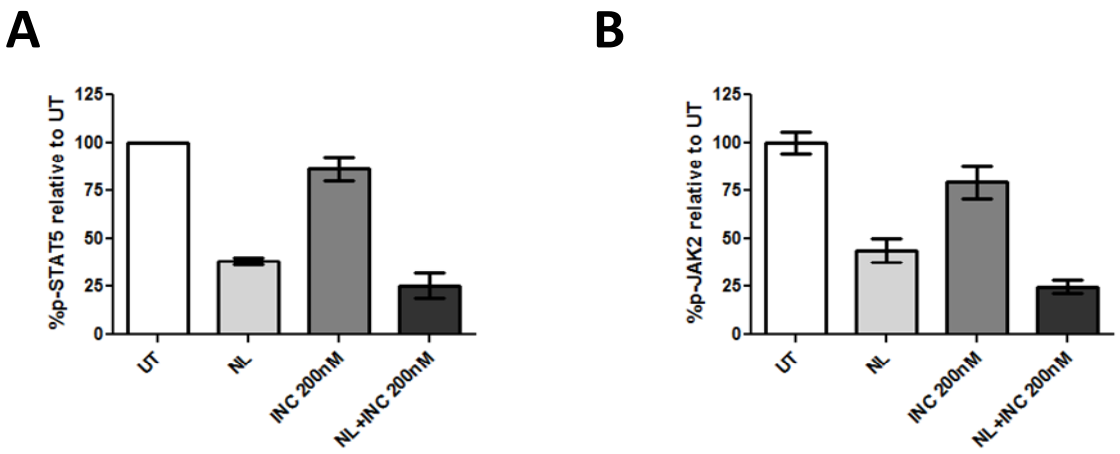


Figure 4-12 p-STAT5 and p-JAK2 levels in CML CD34$^+$ cells following treatment with NL+INC p-STAT5 (A) and p-JAK2 (B) levels were measured by intracellular flow cytometry in 3 CML CD34$^+$ samples after 24 hours of incubation in suspension cultures with NL, INC and NL+INC. Levels of phosphorylation of both proteins were calculated based on MFI of antibody stained samples relative to isotype stained samples. The average of UT values was normalised to 100% and changes following treatment expressed as % change from UT.

4.2.6 Assessment of the effects of NL+INC combination on CML $CD34^+$ cells in the absence of supplemented GFs

All the experiments described above were performed as previously mentioned in SFM+PGF. This culture medium contains, amongst others, two key GFs for myeloid cells, GM-CSF and IL-6, which are able to activate respectively JAK2 and JAK1, the two main kinases inhibited by INC[41]. It was therefore logical to ask if the additive effects seen when combining NL with INC were secondary to INC reducing survival signals emanating from the supplemented GFs. To ensure that any effects due to JAK2 inhibition would not be missed, a concentration of INC of 1,000nM (which is the C_{max} achievable in patients) was used to achieve complete JAK2 inhibition. Moreover given the higher background levels of apoptosis induced over time by the absence of GFs, measurement of cell death and of signalling changes was carried out at earlier time points.

Figure 4-13 shows that both apoptosis and CFC output were not significantly increased by combination treatment compared to NL in the absence of GFs (panel A and B). Similarly p-STAT5 levels at various time points were not further reduced by NL+INC compared to NL alone in the absence of GFs (panel C).

Overall these data suggest that activation of JAK2 by GFs is necessary to observe the additive effects previously reported. In the absence of GFs, JAK2 signals were either absent or under the direct control of BCR-ABL, hence completely abrogated by the high doses of NL used in this experimental setting. Moreover this would also support a model where the previously reported autocrine production of the GFs, such as IL-3 and GM-CSF known to activate JAK2, is not sufficient to activate this kinase in a cell autonomous fashion and particularly when BCR-ABL kinase is fully inhibited.

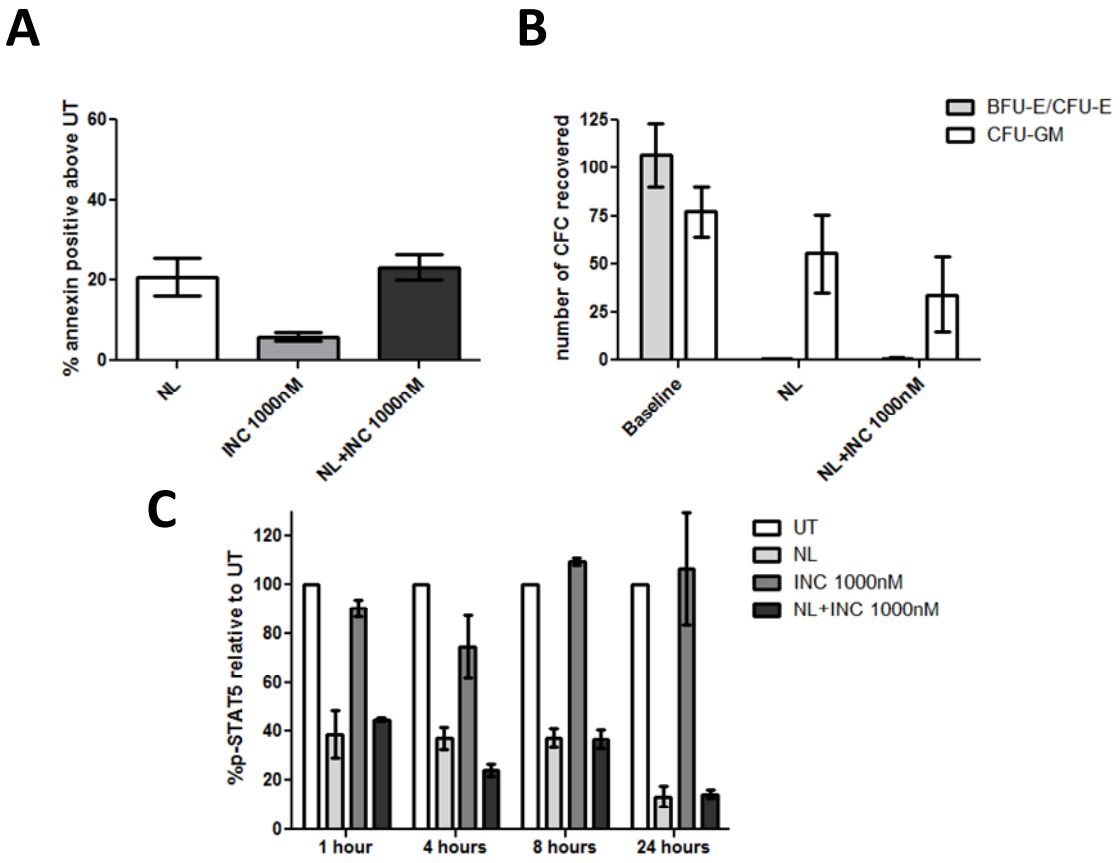


Figure 4-13 Effects of NL+INC combination on CML CD34$^+$ cells in the absence of supplemented GFs

CML CD34$^+$ cells (n=3) were cultured with NL, INC, their combination or left UT in the absence of any supplemented GFs. At 24 hours apoptosis was measured by annexin-V staining (A). Following 72 hours in culture 3,000 cells from each treatment arm were plated in standard CFC assays following drug washout. Number of colonies recovered based on their morphology from each arm and at baseline is plotted in panel B. The number of colonies following 72 hours culture was also adjusted for the expansion of CD34$^+$ cells *in vitro* in each arm relative to baseline. CML CD34$^+$ cells (n=2) were cultured with NL, INC, their combination or left UT in the absence of any supplemented GFs. P-STAT5 levels were measured by intracellular flow cytometry at 1, 4, 8 and 24 hours for each treatment arm. Levels of phosphorylation of STAT5 were calculated as previously described (C).

4.2.7 Investigation of the mechanisms underlying the additive effects seen when combining NL with INC

A recent report from Hantschel *et al* has put in doubt the role of JAK2 in BCR-ABL induced leukaemias. Particularly it has suggested that in these forms of leukaemias, STAT5 is under the direct control of BCR-ABL and therefore JAK2 becomes dispensable. It has also suggested that the additive effects reported by other groups when combining JAK2 inhibitors with TKI are secondary to off-target effects of the JAK2 inhibitor, mainly inhibition of BCR-ABL itself. Therefore in order to clarify the mechanism behind the effects seen in cell biology assays on CML $CD34^+$ cells when combining NL with INC, further experiments were carried out using qRT-PCR to measure STAT5 target gene expression changes following treatment. Moreover this was complemented by an unbiased approach using a proteomic screen of cells treated with NL, INC or their combination. In this set of experiments a higher concentration of INC at 1,000nM was used to ensure that full inhibition of JAK2 was achieved and changes in the genome and proteome could be detected.

4.2.7.1 Investigation of the role of STAT5 in the effects seen when combining NL with INC in CML $CD34^+$ cells

In order to define the best time point at which to perform gene expression analysis a time course analysis of changes in p-STAT5 levels was performed in CML $CD34^+$ cells treated with NL, INC or their combination. These experiments further confirmed that NL+INC rapidly and durably inhibit the activity of this transcription factor to a higher degree than either single agent alone by 8 hours and this effect was persistent up to 24 hours (figure 4-14 A). Moreover no significant apoptosis was induced by the combination treatment at early time points (1, 4 and 8 hours), although a trend towards higher apoptosis started to be present by 24 hours (figure 4-14 B). Based on this it was decided that 8 hours was the best time point for any further signalling analysis. In all the experiments so far described, a high concentration of NL at 5μM was used. It is therefore unlikely that any putative off-target effects of JAK2 inhibitor would contribute to further inhibition of BCR-ABL kinase in this setting. However to exclude this possibility p-CrKL levels were also measured. Western blot analysis of 2 $CD34^+$ CML samples confirmed full inhibition of BCR-ABL kinase activity upon treatment with NL, which was not enhanced by the combination treatment. Moreover no significant changes in p-CrKL levels were observed when cells where treated with INC as a single agent (figure 4-14 C).

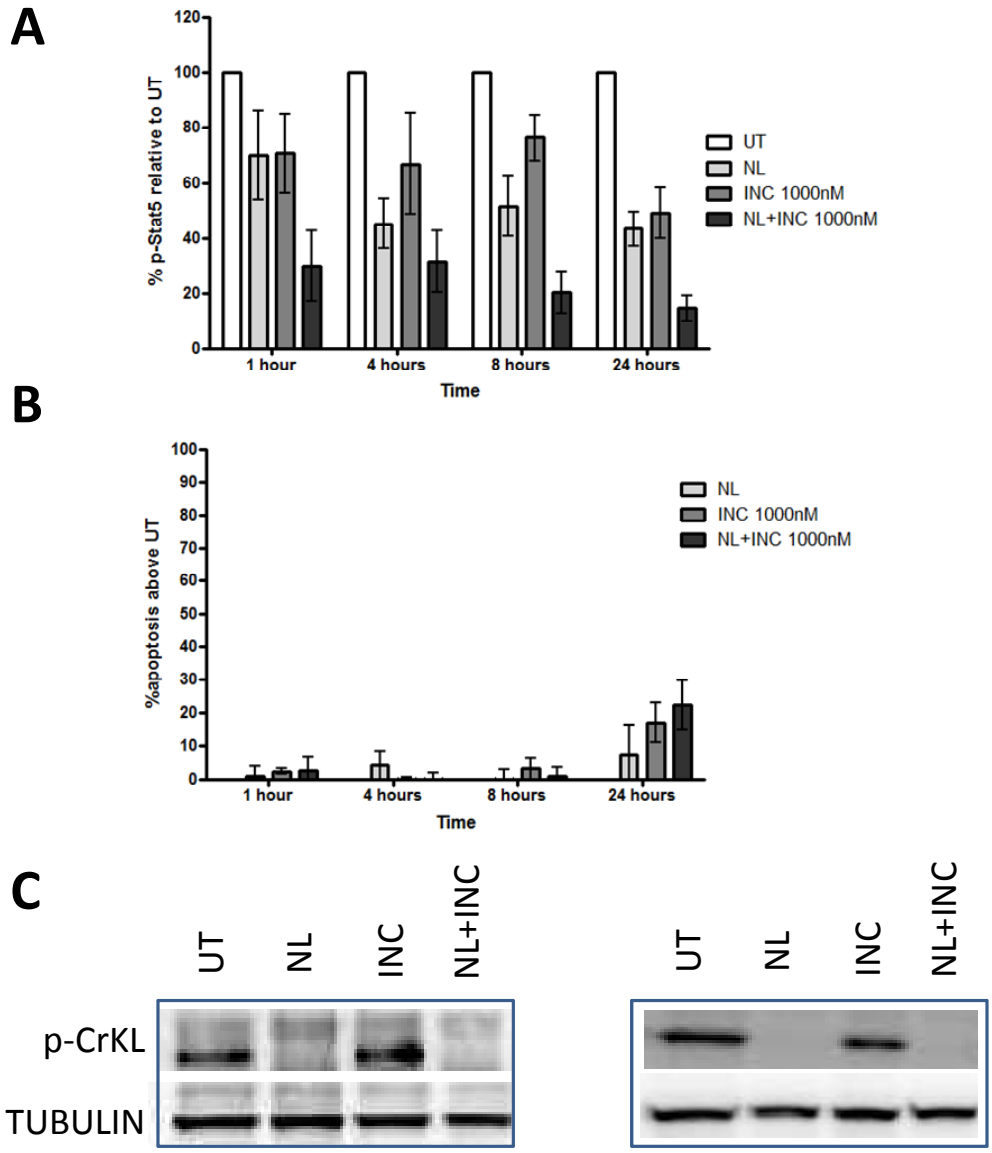


Figure 4-14 Time course analysis of the effects of NL+INC on p-STAT5 levels and apoptosis induction in CML CD34$^+$ cells and effects of NL+INC combination on p-CrKL levels in CML CD34$^+$ cells
CML CD34$^+$ cells (n=3) were cultured with NL, INC, their combination or left UT in SFM+PGF. P-STAT5 levels were measured by intracellular flow cytometry at 1, 4, 8 and 24 hours for each treatment arm. Levels of phosphorylation of STAT5 were calculated as previously described (A). Apoptosis was measured by annexin-V staining at the same time points (B). P-CrKL levels were measured by western blot in CML CD34$^+$ cells (n=2) treated for 8 hours with NL, INC at 1,000nM or their combination. Tubulin was used as a loading control.

In order to look further into the effects caused by the deeper inhibition of p-STAT5 levels by the NL+INC combination, gene expression changes for some known key targets of STAT5 were assessed at 8 hours following treatment. As shown in figure 4-15, compared to NL and INC single agent treatment, the combination treatment caused downregulation of genes known to be positively regulated by STAT5, such as the antiapoptotic gene *BCL-X_L* and those involved in inducing cell cycle and proliferation, such as *cyclin D1*, *D2*, *D3*

or *MYC*. Conversely genes normally negatively regulated by STAT5 were upregulated, such as the AKT pathway negative regulator *PTEN* and the CKI *CDKN1B/p27*. For other known targets of STAT5, such as *MCL1* and *p53*, no changes were observed. It was also noticeable that *TNF-α* and the IL-4 receptor alpha (*IL-4Rα*) were downregulated by NL+INC. TNF-α and IL-4 were amongst 2 of the GFs shown to be upregulated in CML SPC in chapter 3. While STAT6 (which is downstream of JAK1 also inhibited by INC) has been involved in *IL-4Rα* expression, little is known as to the role of STATs in regulating *TNF-α* expression.

Finally two other genes modulated by the NL+INC combination were noticeable: *BCL6* and *ID1*. BCL6 has recently been reported as a key transcription factor for the maintenance of CML LSC[378]. Interestingly in this report the authors link its expression to both the FOXO3a transcription factor and to STAT5. In particular FOXO3a overexpression appears to upregulate *BCL6* expression. STAT5 instead acts as a negative regulator of its expression, whilst STAT5 deletion causes *Bcl6* upregulation. The gene expression analysis shown here therefore appears consistent with such a model, as the deeper reduction in p-STAT5 levels seen following NL+INC correlates with an increased expression of *BCL6*. Moreover it would also suggest this as a potential resistance mechanism to combined TKI+JAK2 inhibitor therapy in CML SPC.

ID1 has also been shown to be controlled by STAT5[379]. It is an inhibitor of basic helix-loop-helix transcription factors which play key roles in HSC self-renewal and differentiation. In particular, ID1 appears to promote self-renewal of HSC[380,381]. More recently two reports have also suggested its potential role in cancer as ID1 has been shown to both promote a myeloproliferative disorder *in vivo*[382] and to maintain colon cancer initiating cells[383]. Its significant downregulation by NL+INC treatment would therefore suggest that it might play a role also in CML SPC survival.

Overall these data show that NL+INC causes a more rapid and deeper inhibition of p-STAT5 activity and this is not secondary to off-target effects of JAK2 inhibitors on BCR-ABL kinase activity. The correlative changes in gene expression of known STAT5 target genes further support a more profound downregulation of STAT5 activity following combination treatment as a key mechanism behind the observed additive effects seen. Moreover it also identifies new potential transcription factors involved in either resistance (*BCL6*) of CML $CD34^+$ cells to this therapeutic strategy or to the overall survival (*ID1*) of CML SPC.

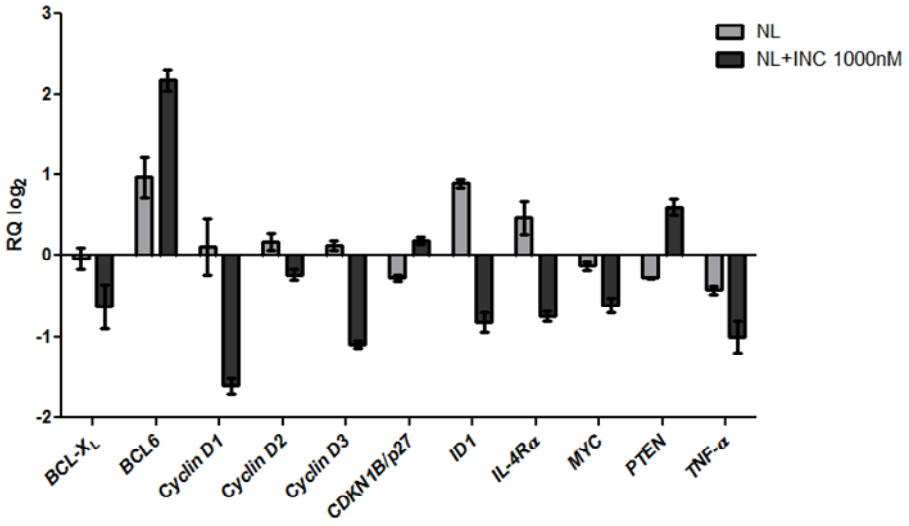


Figure 4-15 Gene expression changes in STAT5 target genes following NL+INC combination treatment

Candidate genes mRNA expression was measured in 2 CML samples following 8 hours culture in SFM+PGF with NL, INC or their combination. Differences in gene expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate genes expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate genes mRNA expression following NL and NL+INC treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the INC cells having a value of 0 in the graph being the calibrator).

4.2.7.2 Investigation of novel mechanisms for the additive effects seen when combining NL with INC using an unbiased proteomic screen

It is often seen in cellular systems that combining two drugs not particularly effective on their own can result in increased cell kill, because of combined effects which could not be appreciated when the drugs were used as single agents. Trying to decipher these effects can help to characterise better the mechanism of action of a drug combination. Moreover it also provides an opportunity to unravel novel pathways and targets which otherwise would have gone unnoticed and could themselves represent potential therapeutic targets. The only way however to achieve this is to use an unbiased approach ideally looking at genome and proteome changes caused by the combination of two drugs. An example of the validity of such an approach has recently been demonstrated in the CML field by a study published by Winter *et al*, which has characterised the synergistic interaction between two multikinase inhibitors, danusertib and BOS, in BCR-ABL transformed cells carrying resistant mutations in the kinase domain. By using a global proteomic and genomic approach they were able to show that reduced MYC activity was the key point of convergence in the synergistic effects seen which had not been appreciated when either inhibitor had been characterised on its own[384]. Following a similar logic it was felt that by interrogating the proteome for any changes caused by the NL+INC combination, it would be possible to identify novel mechanisms behind the combinatory effects seen.

Two CML $CD34^+$ cell samples were therefore treated for 8 hours with NL, INC or their combination and quantitative proteomic changes analysed using mass spectrometry (MS) following isobaric tags for relative and absolute quantitation (iTRAQ) labelling to allow for peptide quantitation (work carried out in collaboration with Dr Emma Carrick and Professor Tony Whetton, University of Manchester, UK). A simplified diagram of the different steps involved in quantitative proteomics using mass spectrometry analysis is presented in figure 4-16.

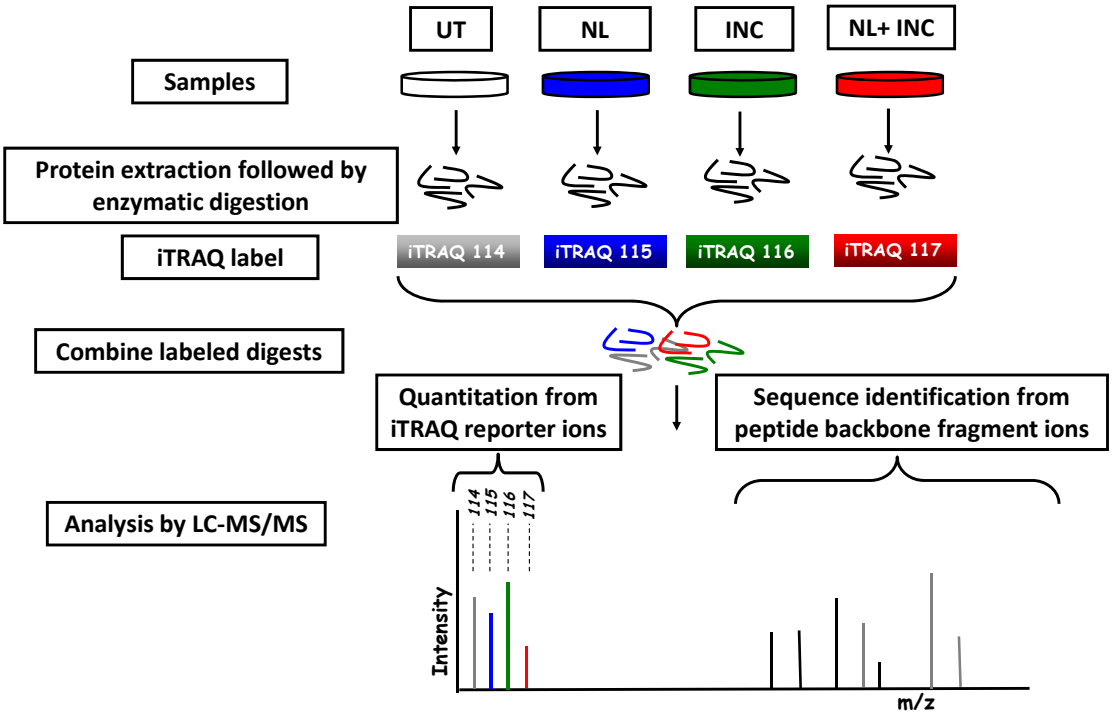


Figure 4-16 Workflow of quantitative proteomic analysis using MS analysis
Proteins were extracted from the 4 different treatment arms at 8 hours in 2 CML CD34$^+$ samples. Following digestion into peptides and labelling with different isobaric stable isotope tags (iTRAQ), the digests are mixed and first separated by liquid chromatography (LC) into different fractions. Thereafter these fractions are fragmented and ionised in the MS and detected as mass to charge ratio (m/z) peptide fragment ions which can then be mapped to the protein to which they belong by searching against public databases. However in addition to the normal fragment ions, the reporter regions of the iTRAQ labels also dissociate in the MS to produce ion signals which provide quantitative information regarding the relative amount of the peptide in the samples.

A total of 12467 peptides were identified which could be matched with confidence to a total of 2686 proteins. Although the original plan was to perform a phosphopeptide enrichment to interrogate also the phosphoproteomic changes (in fact the 8 hour time point had been chosen to capture mainly protein phosphorylation changes), this was not possible because of lack of material (there was only 16µg of protein available per sample while normally 50 to 100µg are necessary for phosphopeptide enrichment). Analysis of the data set produced showed that the combination treatment was able to create a more profound perturbation in the proteome compared to either single agent alone, as more specific protein changes were produced by NL+INC compared to either single agent alone (31 in NL+INC versus 4 in NL arm and 11 in INC arm) (figure 4-17 A left panel). When the common differences in the protein changes between the 2 patient samples were analysed, 29 common changes were identified in the INC versus combination comparison and 8 in the NL versus combination comparison (figure 4-17 A right panel). Given the increased apoptosis seen when combining NL+INC *in vitro* and the fact TKI are standard treatment

for CML, it was felt that the common changes happening in the combination versus NL comparison were more interesting to follow. In fact while TKI are excellent debulking agents in CML, they show little activity against CML LSCs. Therefore focusing on the changes caused by the combination in comparison to single agent TKI alone provided the opportunity to identify novel and relevant survival mechanisms of CML LSC which are not affected by TKI treatment on its own. These could help to explain the increased efficacy of the combination and also identify novel CML LSC specific therapeutic targets. Amongst these 8 proteins there were some interesting candidates, such as the drug transporter ABCC4 and APO-E, both being more deeply downregulated by the NL+INC combination (figure 4-17 B left panel). APO-E however was particularly interesting as it had been shown to be upregulated in CML quiescent stem cells compared to normal quiescent stem cells in a previous gene array carried out in our laboratory[385] (figure 4-17 B right panel).

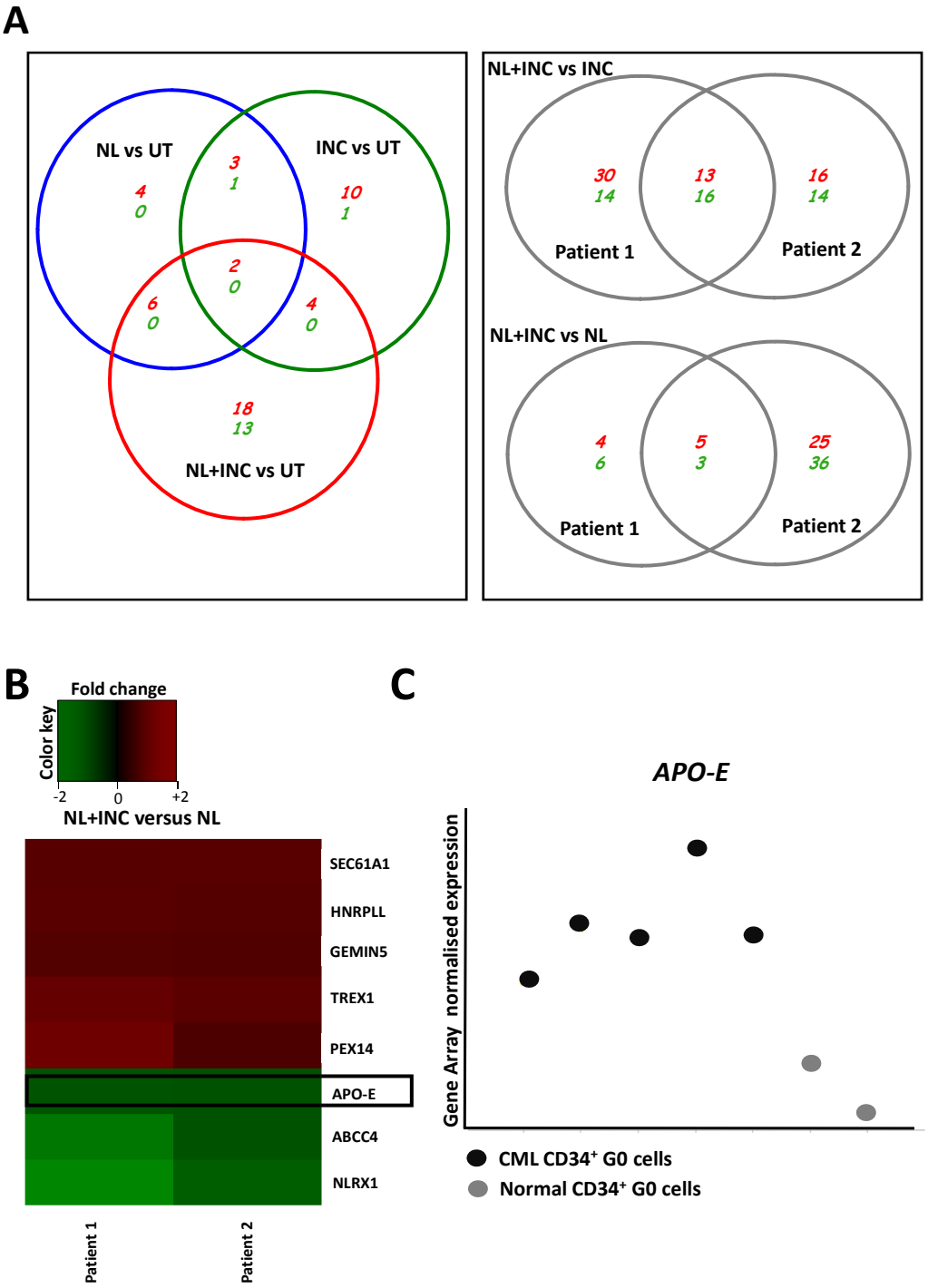


Figure 4-17 Protein changes detected using a quantitative proteomic screen in CML CD34$^+$ cells treated with NL, INC or NL+INC
Venn diagram showing overall protein changes in different treatment arms compared to UT (A, left panel) and common changes between the two patient samples in the combination versus single arm treatment comparison (A, right panel). Heatmap showing the 8 proteins changed in both CML CD34$^+$ samples following NL+INC treatment when compared to NL treated cells from the same samples (B). Levels of *APO-E* expression in 5 CML CD34$^+$ G0 samples versus 2 Normal CD34$^+$ G0 samples from a published gene array carried out in our laboratory (C)[385]. The analysis of the proteomic data set was kindly performed by Dr Lisa Hopcroft, University of Glasgow, UK.

APO-E is a constituent of plasma lipoproteins responsible for cholesterol transport and metabolism, which is mainly produced by the liver, although production by other tissues has also been reported. It appears to play a protective role in response to oxidative stress

as its deficiency is associated with chronic oxidative stress mainly in the brain tissues[386]. APO-E has been reported to promote cell proliferation in serum deprivation conditions, thus suggesting a role as a prosurvival cue[387]. More recently its role in survival and proliferation of cancer cells has also been reported[388,389]. The exact mechanisms behind these effects are still unclear. One possibility that has been proposed is that endogenously produced APO-E might be able to bind members of the LDL receptor family and activate proliferative signalling pathways, such as the PI3 kinase/AKT pathway[388]. It was therefore decided that APO-E was the first candidate to be followed from the proteomic screen.

In order to validate that APO-E levels were indeed higher in CML SPC relative to their normal counterparts, *APO-E* mRNA was measured by qRT-PCR in $CD34^+$ cells from CML and normal samples. *APO-E* was expressed at slightly higher levels in CML $CD34^+$ cells although the difference was not significant ($p=0.11$) (figure 4-18 A left panel). However when changes in both gene expression and protein levels of APO-E were measured following 8 hours treatment with NL, INC or their combination, no differences were observed in the protein levels (figure 4-18 B) and even an increase in its mRNA expression was recorded following both NL and NL+INC treatment (figure 4-18 A right panel).

Overall these data show the validity of an unbiased approach in trying to elucidate the mechanisms underlying the effects seen when combining two drugs together and as a way to potentially identify novel targets. Although APO-E was not validated following the proteomic screen, its high levels of expression in CML cells are potentially interesting and worthy of further investigation. Unfortunately because of lack of time it was not possible to do this and to validate the other candidates and so this will be part of future planned work. Moreover in order to clarify the mechanisms of the combined effects of NL+INC in more detail, it has also been planned to perform a phosphoproteomic screen using a larger number of cells to obtain enough material. This work is currently underway.

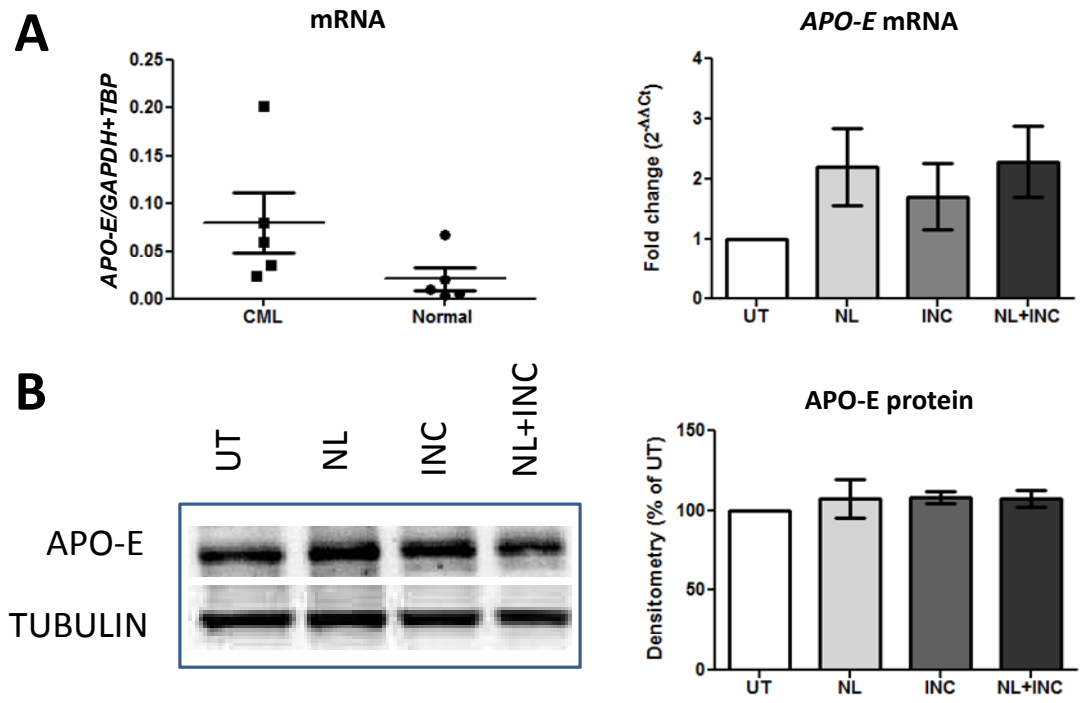


Figure 4-18 Validation of APO-E changes following NL, INC or their combination treatment
mRNA expression of *APO-E* was measured in CML and normal CD34$^+$ samples (n=5) following recovery and overnight culture in SFM+PGF. Levels of expression were calculated using the $2^{-\Delta Ct}$ method after normalisation of *APO-E* mRNA expression levels against the expression levels of a housekeeping gene (*GAPDH* and *TBP*) within each sample (panel A left). *APO-E* mRNA expression was measured in 2 CML CD34$^+$ samples following 8 hours culture with NL, INC or their combination. Differences in *APO-E* expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of its mRNA expression levels against the expression levels of a housekeeping gene (*GAPDH* and *TBP*). Fold change was then plotted relative to UT on a linear scale (with the UT sample having a value of 1 in the graph) (panel A right). APO-E levels were measured by western blot in CML CD34$^+$ cells (n=2) treated for 8 hours with NL, INC or their combination. Tubulin was used as a loading control. Changes in expression levels were quantified using densitometry and expressed as % change relative to UT samples (panel B right). Representative blot from one of the 2 samples (panel B left).

4.3 Summary and future directions

The results of the experiments presented in this chapter using two different JAK2 inhibitors support the conclusion that JAK2 plays a role in survival of CML SPC. These conclusions are supported by the enhanced apoptosis, reduced CFC output and reduced viability consistently seen in CML $CD34^+$ cells when treated with JAK2 inhibitors. The effects were always mild when the JAK2 inhibitors were used as single agents, but became much more dramatic when combined with TKI. The role of JAK2 appears therefore to be prominent especially in the setting of TKI therapy as demonstrated by the fact that both TG and INC in combination with TKI (at concentrations fully inhibiting BCR-ABL kinase) determined a consistent reduction in viability, colony output and proliferation of CML $CD34^+$ cells, including a trend towards reducing the quiescent $CFSE^{max}$ population (INC, figure 4-10) and the $CD34^+$ $CD38^-$ population (TG, figure 4-3) compared to TKI treatment alone. This would suggest that CML $CD34^+$ cells become more reliant on JAK2 kinase when BCR-ABL is fully inhibited and that a combined treatment approach with JAK2 inhibitors and TKI could produce synthetic lethality in CML SPC. These findings are consistent with those recently published by other research groups mainly using non clinically developed JAK2 inhibitors[260,390,391]. Despite all this accumulating evidence on the role of JAK2 in CML, it should however be noted that its role in BCR-ABL positive cells survival has been questioned by a recent report by Hantschel *et al* which shows that BCR-ABL is still able to transform murine BM cells in which *Jak2* expression has been deleted, both *in vitro* and *in vivo*[239]. There are however several possible explanations for the discrepancy in the results observed. It is indeed possible that JAK2's role is dispensable when BCR-ABL is fully active, but that it becomes highly important when BCR-ABL kinase is inhibited. In a truly synthetic lethality model, the role of a therapeutic target can only be evaluated in the context of a combined inhibition of two or multiple targets. Therefore the fact that JAK2 is relevant in the context of TKI does not necessarily contradict its reported dispensable role when BCR-ABL is fully active. It should be noted that sometimes it is necessary to stress a system in order to see a phenotype, for example by combined inhibition of BCR-ABL and JAK2, or secondary and tertiary transplants of BM carrying gene deletions, none of which were performed in the report from Hantschel *et al*. Other technical differences might also help in explaining the differences observed between the Hantschel's report and the results observed here, such as the use of different models (respectively BCR-ABL transduced mouse cells versus primary CP CML cells, with the former likely expressing higher levels of BCR-ABL compared to the latter) and the culture conditions (respectively without and with exogenous GFs). In this respect it is

interesting to note that the actual debate on the role of JAK2 in CML is reminiscent of the old debate on the role of IL-3 in BCR-ABL induced leukaemias *in vivo,* which in any case is also biologically linked (given that IL-3 does signal via JAK2), where completely opposite results, both perfectly explainable, were obtained based on different experimental conditions (see section 1.3.6.2). Considering these points will help to reconcile the discrepancy between the results reported in this chapter and those reported by Hantschel *et al*.

To assess the mechanisms behind the activation of JAK2 in CML SPC and test the hypothesis that autocrine IL-3 and GM-CSF production is a relevant and cell autonomous mechanism supporting JAK2 activation and CML $CD34^+$ cell survival, experiments in the absence of exogenous GFs were performed. These showed that in this setting JAK2 inhibition had only very mild effects on survival of $CD34^+$ cells and did not synergise with TKI therapy and support the conclusion that autocrine production of GFs (i.e. GM-CSF and IL-3) by CML $CD34^+$ cells is not sufficient to activate JAK2 in a cell autonomous and BCR-ABL kinase independent fashion. In this respect it is worth noting that also in the original report showing autocrine IL-3 production by primary CML $CD34^+$ cells, when the cells were plated in bulk cultures with neutralising anti-IL-3 antibodies only a relatively low reduction of proliferation was observed and only when high concentrations of neutralising antibody were used[270]. There are several possible explanations for this result:

1) Both our data and published literature[270] show that the level of autocrine production of IL-3 and GM-CSF is low and often inconsistent between patient samples which might preclude the observation of a truly autocrine effect in *in vitro* cultures.

2) If autocrine GFs production is a feature of only few selected cells, it is possible that in bulk cultures a truly autocrine effect cannot be observed because of high local concentrations of GFs around individual autocrine cells.

3) The high concentration of NL used caused already marked effects on cell death and signalling in the absence of GFs which would have precluded observing any additive/synergistic effects of JAK2 inhibitors in the setting of TKI therapy. A titration of NL in this experimental setting would have probably been appropriate and is planned in future work.

4) Both inhibitors used are also capable of targeting other members of the JAK family so it is theoretically possible that some of the effects seen were also secondary to inhibition of other JAK family members activated in the presence of exogenous GFs used in our standard culture media. For example IL-6, which is normally present in the GFs mix used in the standard media, is known to activate JAK1 together with JAK2[376]. In the absence of GFs the only JAK possibly active would have been JAK2 via autocrine IL-3 and GM-CSF and might not have been enough to replicate the same effects seen when other GFs and possibly other JAK proteins were active.

Overall the lack of effects in the absence of supplemented GFs are consistent with other reports using CML $CD34^+$ cells suggesting that the main role of JAK2, independent of BCR-ABL, is to relay survival signals by exogenous GFs[260,390,391]. This observation helps also to resolve, to an extent, the discrepancy in the results observed with the findings from Hantschel *et al* as all their *in vitro* transformation assays were performed in the absence of supplemented GFs.

Based on the above findings, it is no surprise that the observed mechanism of action for JAK2 inhibitors in combination with TKI in the presence of exogenous GFs is a more profound inhibition of JAK2/STAT5 activity, as shown by the correlative changes in both p-JAK2 and p-STAT5 levels with combined treatment. Moreover the correlative gene expression changes of STAT5 target genes further support this conclusion, while also providing some mechanistic explanations for the effects observed with combination treatment, as proliferation inducing genes such as *cyclin D1*, *D2* and *D3* and *MYC* and the antiapoptotic gene *BCL-X_L* were all downregulated, while negative regulators of cell cycle and proliferative signals, such as *CDKN1B/p27* and *PTEN* were upregulated. In this respect it is worth noting that in the Hantschel's report off-target inhibition of BCR-ABL kinase was observed by several JAK2 inhibitors resulting in reduced phosphorylation of STAT5 which was considered the likely explanation for the JAK2 inhibitor efficacy in killing BCR-ABL positive cells. However in the same report it was noted that such off-target effects were only seen when TG was used at higher than 500nM and INC at higher than 20μM, which are both well above the concentrations used in the experiments described in this chapter. Moreover the high TKI concentration used in the experiments described in this chapter achieved maximal BCR-ABL kinase inhibition, which makes it highly unlikely that further inhibition of the same kinase activity could be caused by off-target effects of the JAK2 inhibitors, as shown by changes in p-CrKL levels following NL, INC and their combination treatment (figure 4-14 C). Finally the close correlation observed between the

phosphorylation changes in JAK2 and STAT5 (figure 4-12), further supports JAK2 inhibition as being responsible for the further reduction in STAT5 activity. Therefore it can be excluded that the effects on STAT5 activity seen in the reported experiments were all secondary to BCR-ABL kinase inhibition, but they were rather secondary to JAK2 inhibition.

It should be noted however that some of the genes whose expression was modulated by INC are not specifically or uniquely under the control of STAT5 while for others, such as *TNF-α* and *IL-4Rα*, the evidence of a control by STAT5 is lacking. This would suggest that other pathways besides STAT5 can be inhibited by JAK2 itself, which is consistent with the discrepancy seen in the phenotype of *Jak2* and *Stat5* KO mouse models. Recently the nuclear localisation of JAK2 has been shown in both JAK2 positive cell lines and BCR-ABL positive K562 cells and was associated with a STAT5 independent effect of JAK2 on chromatin structure and gene expression through direct phosphorylation of histone 3[362]. Also literature published in the CML field in the past and more recently has suggested possible STAT5 independent effects for JAK2, including activation of MYC[240], phosphorylation of BCR-ABL on its key residue tyrosine 177[367] (which is central for its transforming capacity through its ability to activate both the RAS/MAP kinase and the PI3 kinase pathways[210-212]) and activation of β-catenin[369].

Another important point to note is that both TG and INC are able to inhibit other JAK kinases (JAK1 and JAK3) and therefore some of the effects seen could be secondary to inhibition of these other kinases. In this respect, it is worth noting that other reports on the efficacy of JAK2 inhibitors in BCR-ABL positive cells have showed inhibition of STAT3 activity (also a downstream target of both JAK1 and JAK3) as a possible mechanism of action for these agents[390,391]. In order to assess more precisely the relative contribution of STAT5 and of other JAK kinases in the effects seen on CML $CD34^+$ cells following JAK2 inhibitor treatment, inhibition of STAT5 and JAK2 expression in CML $CD34^+$ cells using RNA interference techniques could be employed. Although this approach was explored, adequate knockdown was never achieved and therefore results were not presented in this chapter. Trying to optimise this approach is part of the future work plans. Nevertheless the consistent results obtained with two different JAK2 inhibitors (who had different inhibitory effects on other JAK kinases) in terms of reduction of p-STAT5 levels, together with the correlative changes in p-JAK2 levels and gene expression are highly suggestive that inhibition of the JAK2/STAT5 axis plays a central role in the effects observed. In summary based on the presented experimental data, it can be concluded that enhanced inhibition of

STAT5 activity by JAK2 inhibitor and TKI combination can be considered the major mechanism behind the effects seen, although interference of the JAK2+TKI combination with other yet poorly characterised signalling pathways is also possible (figure 4-19).

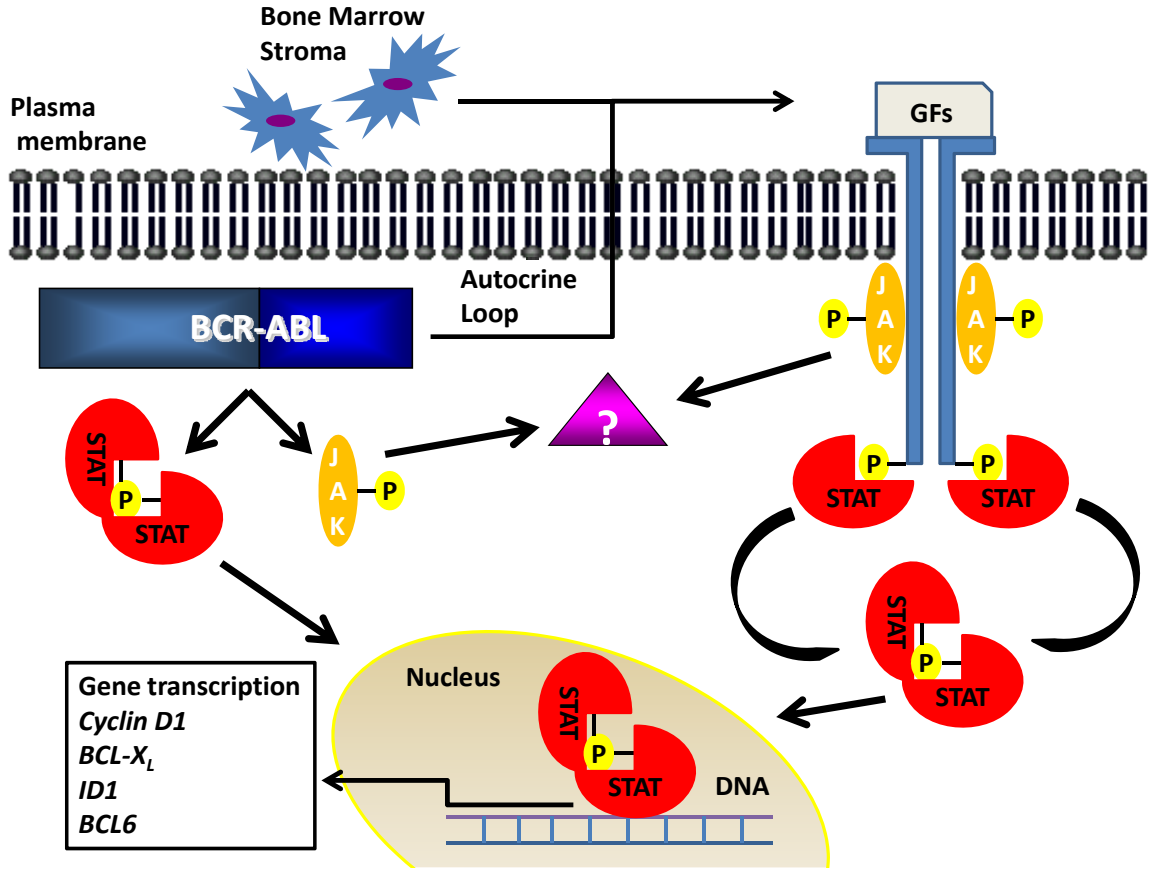


Figure 4-19 JAK2 role in CML SPC
JAK2 can be activated both directly by BCR-ABL kinase and via autocrine and paracrine GFs. Upon activation it relays its signals through STAT5 and possibly other as yet unknown signalling pathways. Inhibiting both BCR-ABL kinase and JAK2 kinase activity in CML SPC has an additive effect through enhanced downregulation of STAT5 signalling and also possibly through interference of other pathways.

In an attempt to explore any novel mechanisms underlying the observed efficacy of the NL+INC, an unbiased proteomic screen was used. The value of such an approach, particularly in being able to identify novel mechanisms of action and targets of combined drug therapies, has already been shown[384]. Unfortunately due to the lack of material, the original plan to perform a phosphoproteomic screen had to be abandoned and only a proteomic screen was performed. However its preliminary analysis is in support of the combination treatment causing changes in the proteome not brought about by either single agent alone with some potentially interesting candidates highlighted. An unbiased screen could therefore shed more light on the mechanisms of action for the TKI+JAK2 inhibitors combination treatment. Future work (already in part underway) includes therefore

completing the validation of the proteomic screen performed (which could not be completed due to lack of time) and extending it further to include the phosphoproteome.

An interesting aspect to discuss in more detail is whether or not the data from the presented work support an effect of the TKI+JAK2 inhibitor combination on the most primitive CML LSCs. Various aspects of the data point towards the combination treatment being able also to eradicate the most primitive CML LSC. Both the phenotypic analysis of the viable cells following TG+TKI treatment and the CFSE experiments with INC suggest an enhanced effect of the combination treatment on $CD34^+$ $CD38^-$ cells and $CFSE^{max}$ cells, both of which are known to be enriched for primitive LSC. Moreover the *in vivo* data produced by our collaborators in Vancouver are also supportive of an enhanced effect of the combination treatment on most primitive LSCs, as mouse engraftment with $CD34^+$ cells following combination treatment was consistently reduced. In this respect the marked inhibition of *ID1* gene expression (a gene known to be involved in the maintenance of HSCs and also other CSCs[380,381,383]) is also supportive of an effect on CML LSC survival by the combination treatment. Conversely it has to be said that none of the *in vitro* tests performed is a true readout for LSC activity and the gold standard to assess an effect on LSC remains the *in vivo* work. Moreover the effects on *BCL6* gene expression suggest that the combination treatment might also upregulate genes involved in LSC maintenance. BCL6 was recently shown to be central to CML LSC resistance to TKI and to have a critical role in self-renewal signals of CML LSC[378]. It is therefore possible that the net effect of all these signalling changes is what will eventually determine whether or not the combination treatment has an effect on the most primitive LSCs. Although suggestive of an effect at LSC level, on the basis of the data presented, it is not possible to conclusively say that JAK2 inhibitors in combination with TKI eradicate the most primitive LSCs and further work is necessary to prove this starting from more *in vivo* assays which are currently being performed by our collaborator in USA, Professor Ravi Bhatia (City of Hope, Duarte, California, USA).

The toxicity to normal haemopoiesis of a therapeutic strategy targeting JAK2 is an obvious concern based on the essential role of JAK2 for erythropoiesis as suggested by the embryonic lethal phenotype of the *Jak2* KO mouse. Moreover a recent report by Traer *et al* has highlighted these concerns further and suggested that targeting JAK2 in CML might not be possible following experimental work showing toxicity to normal haemopoiesis with TG used at concentrations of 250nM[390]. The presented experimental work shows however that, despite a degree of toxicity on normal $CD34^+$ cells, a strategy combining

JAK2 inhibitors and TKI could preferentially eradicate CML compared to normal $CD34^+$ cells when a carefully selected concentration of the JAK2 inhibitor is chosen (for example 100nM of TG was used in the presented experiments). A speculative explanation for this would be that the higher levels of CSF2RB in CML compared to normal $CD34^+$ cells (see 3.1.3 and 3.2.1) would render these cells more sensitive to JAK2 inhibition. The above conclusions are obviously based only on *in vitro* experiments and will require further confirmation by *in vivo* studies which are currently being performed by our collaborators using a combination of NL+INC in a CML transgenic mouse model. With regards to the safety of JAK2 inhibitors in the adult, it is also reassuring that INC has been used as a single agent for the treatment of patients with myelofibrosis, with no severe BM toxicity, which points towards the possibility that JAK2 inhibition in an adult *in vivo* might be less toxic than *in vitro* or during embryonic development as seen in the *Jak2* KO mice. Inhibition using a potent pharmacological inhibitor could in fact be less detrimental to normal haemopoiesis than gene deletion while it is also often seen that the effects of targeting a gene in adult stem cells are different to the effects of targeting the same gene during embryonic development. However caution has to be exercised in drawing any conclusions based on this clinical observation as the potential toxicity to normal haemopoiesis of a combined TKI+INC treatment in CML patients might be different.

In conclusion, given the effects seen with the combination treatment on CML $CD34^+$ cells, including $CFSE^{max}$ cells and the $CD34^+$ $CD38^-$ population, it is plausible that a combination treatment with TKI and already clinically developed JAK2 inhibitors in CML patients might help in reducing and potentially eradicating minimal residual disease. However given the fact that a definite effect on the most primitive LSC is still lacking and the potential concerns regarding its toxicity, it might not be advisable at this stage to support an upfront treatment of newly diagnosed CP CML patients with this combination treatment or in patients with persistent disease. Conversely, its use in patients where the clinical need is higher can be supported at this stage, such as CP CML patients who show resistance to TKI in the absence of BCR-ABL dependent resistance mechanisms and even in patients with advanced phase disease. Although its efficacy in this latter setting was not formally tested in the experiments presented, it has been reported by other groups[367] and could again be postulated above all in the absence of BCR-ABL kinase mutations or gene amplification.

5 Results (III) Investigation of the role of autocrine TNF-α production in the survival and proliferation of CML CD34$^+$ cells

TNF-α has complex and varied effects in normal haemopoiesis which have already been discussed in detail in the Introduction (see section 1.1.2.3). Its role appears to be highly dependent on the cell context, its concentration and the presence of other GFs involved[135,136] with both proliferative and inhibitory effects being reported. TNF-α is able to stimulate the proliferation and expansion of the more primitive human haemopoietic cells (CD34$^+$ CD38$^-$) *in vitro,* particularly when the latter are cultured in the presence of high concentrations of GFs, such as IL-3 and GM-CSF [135,137-139]. These proliferative effects are however also associated with a more rapid terminal differentiation of HSPC towards the monocytic lineage leading to inhibition of granulocytic differentiation and growth suppression of mature granulocytes. As a result the effects of TNF-α on more mature and committed granulocytic precursors are rather inhibitory than stimulatory[140,141]. The effects of TNF-α on haemopoiesis are both direct and indirect as TNF-α can also stimulate HSPC and other cells from the microenvironment to produce GFs involved in HSPC proliferation and differentiation (such as GM-CSF) [145,146] or to upregulate GF receptor expression[147] (i.e. CSF2RB).

Similarly the role played by TNF-α in cancer biology is also very complex. Originally discovered as a cytotoxic to cancer cells[392] (hence its name), TNF-α soon proved to be more than that. Small amounts of TNF-α are constitutively produced by many malignant cells, in contrast to their normal counterparts. Moreover excess TNF-α is often produced by other immune cells as a result of the inflammatory reactions surrounding tumour cells. Regardless of its source TNF-α can contribute to tumourigenesis, both indirectly by creating a tumour-supportive inflammatory microenvironment and through direct effects on malignant cells[393]. For example, in colitis associated cancer, TNF-α, produced by inflammatory cells, plays a clear supportive role mainly through enhanced expression of several inflammatory GFs, which in turn activate survival and proliferative signals in the cancer cells[394,395]. On the other hand autocrine TNF-α production has been shown to support lymphoma cell proliferation directly[396]. In both circumstances a central role is played by the TNF-α mediated activation of NFκB[397], a central transcription factor for both prosurvival and proinflammatory signals, which is often constitutively active in TNF-α

producing cancers. Inhibition of the NFκB pathway, through either anti-TNFα treatment or genetic targeting, is coupled with induction of apoptosis in several cancers associated with high TNF-α activity[397,398]. One of the key components of NFκB survival signals is its ability to activate several antiapoptotic proteins, such as BCL-X_L, survivin and the IAP family, which includes IAP1, IAP2 and XIAP[399]. IAPs (particularly IAP1 and IAP2) have been shown to be activated by NFκB and in turn activate NFκB through a positive feedback loop. Moreover they are able to block caspase-8 and any proapoptotic signal relayed by TNF-α so that in their presence the net output of TNF-α signalling is skewed towards promoting survival and proliferation of its target cells[158,161]. Therefore in the context of a cancer cell which has constitutively active NFκB and high levels of expression of IAPs, autocrine and paracrine TNF-α signalling becomes a potent prosurvival and proliferative cue[400]. Interference with this mechanism by using inhibitors of IAPs (so called SMAC mimetics) is able to redirect TNF-α signalling towards induction of apoptosis. Such an approach has been shown to be successful in solid cancers and is particularly effective in those producing autocrine TNF-α as it turns its signals into cell death promoting signals[401-404]. Again from the above evidence it appears that TNF-α signalling in cancer acts as a double edged sword depending on the cell context.

Haemopoietic cancers, including BCR-ABL positive leukaemias, are capable of autocrine TNF-α production[405]. Moreover they also show high levels of expression of IAP proteins[406] and a therapeutic strategy targeting IAPs is currently being investigated in some of these diseases[407]. It is therefore intriguing to speculate that autocrine production of TNF-α by haemopoietic cancer cells could indeed act as a prosurvival signal too. However investigation of TNF-α role in these leukaemias, including CML, is lacking. The first reports published several years ago suggested a role for TNF-α in inducing CML cell kill. It has to be noted however that in those reports most of the experiments were carried out on unselected MNC fraction from CML patients and often using very high concentrations up to 100ng/mL[405,408]. More recently Zhang *et al* reported higher levels of TNF-α in the BM of BCR-ABL induced transgenic mice compared to wild type controls. In functional assays in the same report it was also shown that stem cells from BCR-ABL induced mice displayed a growth advantage compared to normal control stem cells when cultured in the presence of physiological TNF-α concentrations[276]. The experiments shown in 3.1.3 demonstrate that both primary CML $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ cells express higher levels of *TNF-α* mRNA compared to their normal counterparts. Higher levels of expression in CML $CD34^+$ cells compared to normal $CD34^+$ cells were also confirmed at the protein expression level. Finally autocrine production of TNF-α by CML SPC appeared not to be

significantly modulated by BCR-ABL kinase activity. Based on these observations and the evidence coming from the literature regarding TNF-α's role in other cancer systems, it was decided to test the hypothesis that autocrine TNF-α could act as a survival signal in CML SPC. Further support of this hypothesis was provided by a recent report proving that such a mechanism is active in BCR-ABL negative myeloproliferative disorders[409]. In this chapter therefore the aim was to answer the following research questions:

1) Does autocrine TNF-α act as a prosurvival signal for CML SPC?

2) Which mechanisms and pathways are modulated by autocrine TNF-α to determine its effects on CML SPC survival and proliferation?

3) Assuming autocrine TNF-α is acting as a prosurvival signal in CML SPC, can a therapeutic strategy targeting TNF-α be used against CML SPC? What effects would this have against normal HSPC? Could it be combined with TKI therapy?

In order to answer these research questions, a small molecule inhibitor of TNF-α was used alone and in combination with exogenously supplied TNF-α used to rescue the phenotype and further clarify its exact role in CML SPC biology. Protein and gene expression assays were used to investigate the signalling pathways and the mechanisms for the effects seen following TNF-α inhibition and stimulation. Toxicity of TNF-α inhibition on normal SPC was assessed and the effects of combining TNF-α inhibition with TKI therapy in CML SPC cells were evaluated. Finally confirmatory experiments using a TNF-α blocking antibody were carried out.

5.1 Investigation of the role of autocrine TNF-α in CML and normal CD34$^+$ cells using a small molecule TNF-α inhibitor

5.1.1 Assessment of IC_{50} for TNF-α inh in BCR-ABL positive cell lines and primary CML CD34$^+$ cells

As a first step before proceeding with further investigations into the role of TNF-α in CML SPC survival, dose finding experiments for the small molecule TNF-α inh to be used were performed. This compound (originally described in a report in Science in 2005[410]) is a cell-permeable indolyl-chromenone compound that rapidly inactivates TNF-α by non-covalently binding to the TNF-α trimer and promoting subunit dissociation and preventing binding to its receptor (figure 5-1 A). As a result it would be able to inhibit soluble or membrane bound or receptor bound TNF-α. Being a small molecule it would also be capable of blocking any intracellular TNF-α. For these reasons it was selected over other now established anti-TNF-α agents, such as the blocking antibodies (Infliximab) and the decoy receptors (Etanercept)[411]. By virtue of their nature, neither of these compounds would necessarily be able to bind to receptor bound or intracellular TNF-α. Moreover a small molecule inhibitor would represent a more attractive option to follow on clinically compared to blocking antibodies and decoy receptors which both require parenteral administration.

A first set of experiments was carried out in the BCR-ABL positive cell line KCL22 as this cell line had been shown to produce autocrine TNF-α (see figure 3-1) (figure 5-1 B). Based on reduction in viable cell numbers and induction of apoptosis in KCL22, a range of concentration of the TNF-α inh between 2 and 4μM was chosen for further testing in primary CML CD34$^+$ cells. As shown in figure 5-1 C, 3μM of TNF-α inh reduced CD34$^+$ cells viable cell number just below 50% relative to UT with evidence of induction of apoptosis. Based on these results a concentration of 3μM was used in all subsequent experiments in primary CML CD34$^+$ cells.

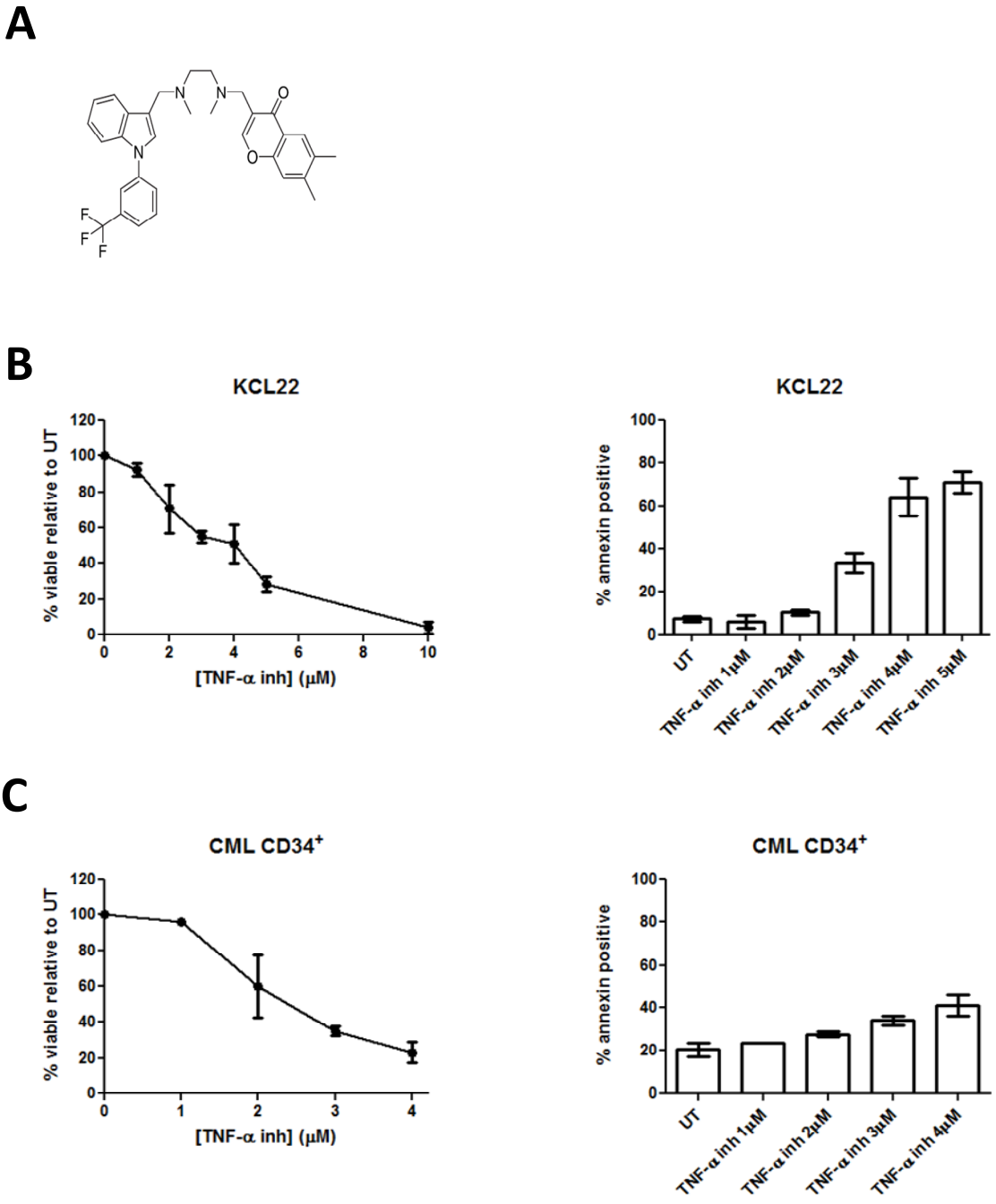


Figure 5-1 Molecular structure of TNF-α inh and identification of IC_{50} for TNF-α inh in the BCR-ABL positive cell line KCL22 and CML $CD34^+$ cells
TNF-α inh (A) was tested at increasing concentrations on KCL22 grown in $RPMI^{++}$ for effects on viability and apoptosis following 48 hours of incubation. Experiments were performed in triplicate (B). CML $CD34^+$ cells (n=2) were cultured in SFM+PGF with increasing concentrations of TNF-α inh and viability and apoptosis were measured at 48 hours (C). Viability was measured by counting cells using trypan blue dye exclusion method. Apoptosis was measured by annexin-V/7AAD staining.

5.1.2 Assessment of the effects of TNF-α inhibition and stimulation in inducing apoptosis and proliferation of CML $CD34^+$ cells

In order to assess the role of TNF-α in survival and proliferation, CML $CD34^+$ cells were cultured in the presence of TNF-α inh, with or without, exogenously added TNF-α. Different concentrations of TNF-α were used in these rescue experiments with very similar results obtained with both 0.1 and 1ng/mL. Therefore for ease of presentation only the results obtained with TNF-α 1ng/mL will be shown. In a first set of experiments apoptosis was measured at different time points. TNF-α inh induced 10-15% apoptosis above UT cells and this could be rescued by exogenous TNF-α (figure 5-2 A). In order to assess the effects of TNF-α inh on both proliferative and quiescent CML $CD34^+$cells, CFSE staining was used. Again TNF-α inh reduced overall proliferation of CML $CD34^+$ cells relative to UT cells. Moreover it also caused cell death as shown by the significant reduction in number of $CD34^+$ cells recovered within each division starting from division 1 ($p<0.001$ for divisions 1, 2 and 3) and an overall significant 43% reduction in the total $CD34^+$ recovered cells compared to UT ($p<0.05$). Although a reduction in the recovered cells within the $CFSE^{max}$ population was observed with TNF-α inh, this was not statistically significant. However a definite effect on the survival of this population was present as a significant induction of apoptosis within the $CFSE^{max}$ population was observed ($p<0.05$). Moreover all these effects were partially rescued by exogenous TNF-α (figure 5-2 B and C).

Overall these data show that autocrine TNF-α supports CML SPC survival and proliferation with some effects seen also on the quiescent stem cell compartment.

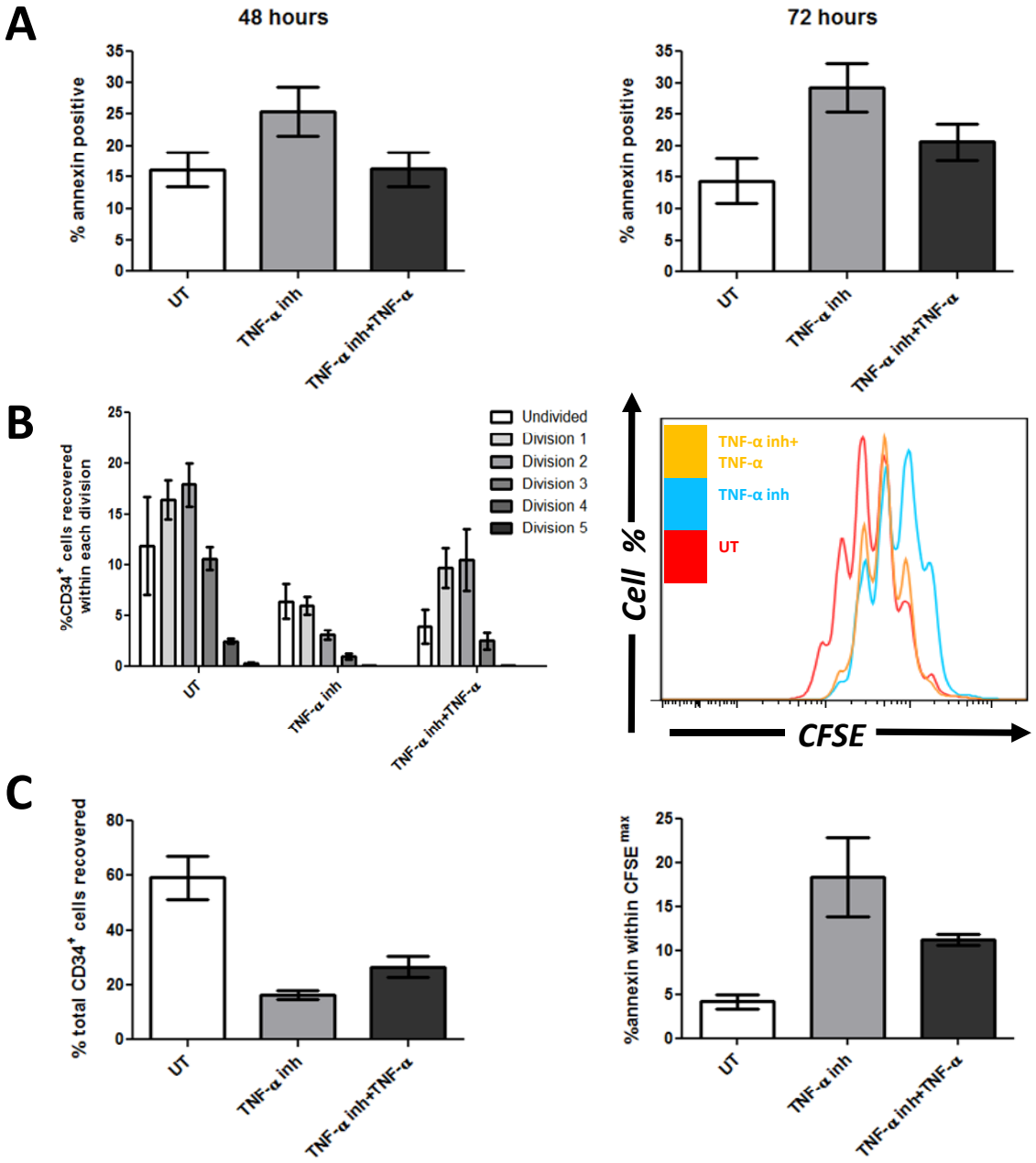


Figure 5-2 Effects of autocrine TNF-α in CML CD34$^+$ cells survival and proliferation
CML CD34$^+$ cells (n=5) were cultured in SFM+PGF with TNF-α inh with or without TNF-α or left UT. Apoptosis was measured by annexin-V/7AAD staining at 48 and 72 hours (A). CML CD34$^+$ cells (n=3) were CFSE stained and then cultured for 72 hours with TNF-α inh with or without TNF-α or left UT. At 72 hours cells from each arm were co-stained with annexin-V and flow cytometry analysis performed. A representative plot is shown in B, right panel. Percentages of starting CD34$^+$ cells recovered within each division (B) and in total (C, left panel) following treatment were calculated as explained in 2.3.2.3. Percentage apoptosis within CFSE^{max} was measured by gating on the population double positive for maximal CFSE expression and annexin-V staining (C, right panel).

5.1.3 Assessment of the effects of TNF-α inhibition and stimulation in inducing apoptosis and on proliferation of normal CD34$^+$ cells

Although normal CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells produce less autocrine TNF-α than their CML counterparts (see figure 3-4), they are still able to produce small amounts. In order to assess the role of autocrine TNF-α in normal HSPC biology, similar experiments to the one performed on CML CD34$^+$ were performed on normal CD34$^+$ cells using the same concentrations of both TNF-α inh and TNF-α. In contrast to CML SPC, normal HSPC did not undergo apoptosis when exposed to TNF-α inh (figure 5-3 A). Moreover no significant changes in the normal CD34$^+$ cell proliferation and cells recovered within each division and in total were observed following TNF-α inh treatment, except for a slight reduction in recovered cells in the later divisions (figure 5-3 B). Adding exogenous TNF-α did not change any of these results. Taken together these results show that autocrine TNF-α does not play a significant role in normal CD34$^+$ cell survival and proliferation.

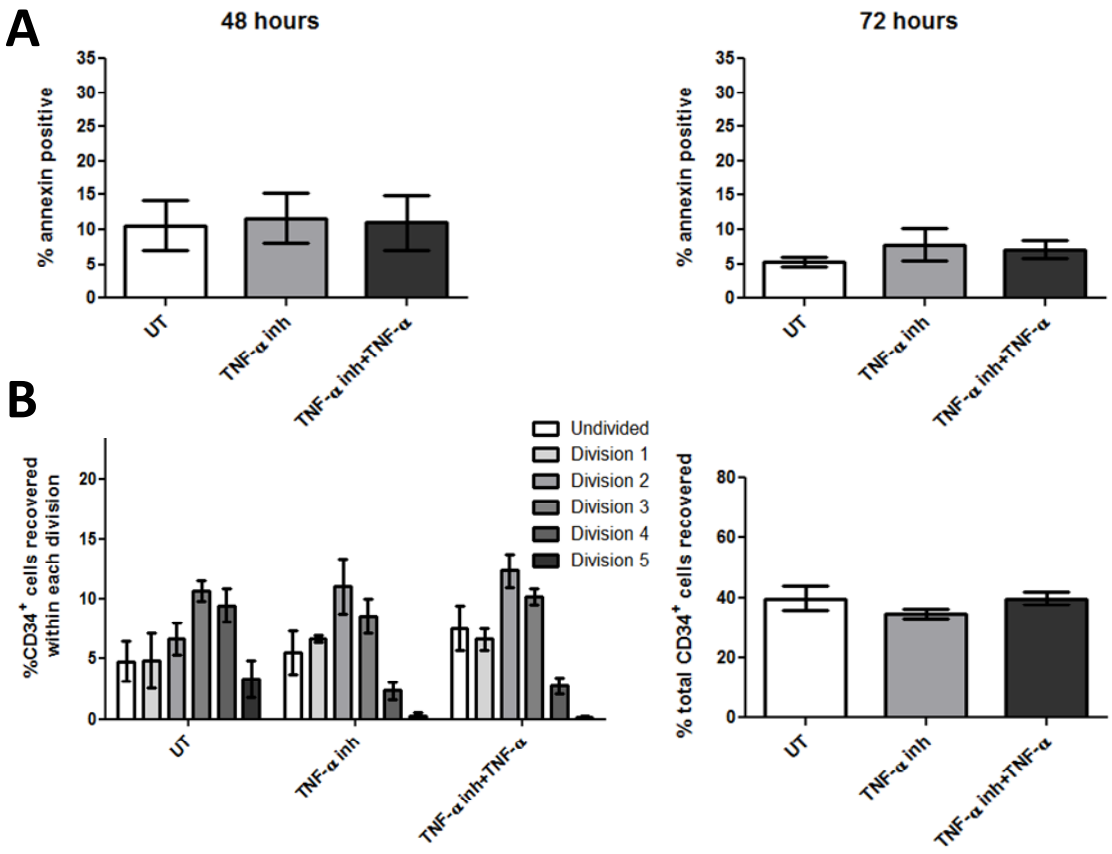


Figure 5-3 Effects of autocrine TNF-α in normal CD34$^+$ cells survival and proliferation
Normal CD34$^+$ cells (n=3) were cultured in SFM+HiGF with TNF-α inh with or without TNF-α or left UT. Apoptosis was measured by annexin-V/7AAD staining at 48 and 72 hours (A). Normal CD34$^+$ cells (n=3) were CFSE stained and then cultured for 72 hours with TNF-α inh with or without TNF-α or left UT before flow cytometry analysis. Percentage of starting CD34$^+$ cells recovered within each division (left panel) and in total (right panel) following treatment was calculated as explained in 2.3.2.3 (B).

5.1.4 Investigation of the mechanisms underlying the survival and proliferative effects of autocrine TNF-α in CML CD34$^+$ cells

5.1.4.1 Analysis of baseline expression of key antiapoptotic genes in CML CD34$^+$ cells

Evidence from the literature, discussed in the chapter introduction, suggests that for TNF-α to be able to relay prosurvival rather than apoptotic signals, NFκB dependent survival signals need to be active and in particular antiapoptotic proteins such as BCL-X_L, survivin and the IAP family need to be expressed[399]. Because of the presence of the BCR-ABL oncogene, CML SPC have a constitutive level of activation of NFκB[252,253]. Therefore to confirm that the key antiapoptotic genes involved in NFκB signalling were expressed in CML CD34$^+$ cells, gene expression analysis was carried out in CML CD34$^+$ cells and compared to their normal counterparts. If indeed the expression of these key antiapoptotic genes was confirmed to be high in CML CD34$^+$ cells, it would provide support to the idea that autocrine TNF-α can act as a prosurvival cue in this specific cell context. As shown in figure 5-4, all the antiapoptotic genes tested (except *XIAP*) had roughly the same level of expression if not higher (*BCL-X_L*) than the housekeeping gene used thus confirming their expression. Moreover for *IAP2*, *BCL-X_L* and *MCL1* (another key antiapoptotic gene although not a direct NFκB target) expression levels were higher in CML than normal, with the difference approaching statistical significance for *BCL-X_L* and *MCL1* (p=0.09) (A). For the other genes tested (*IAP1*, *XIAP* and *survivin*) no difference was detected at the gene expression level between normal and CML CD34$^+$ cells (B). However it should be noted that normal CD34$^+$ cells were grown in SFM+HiGF which might stimulate expression of some of these genes. Moreover a difference at the protein level cannot be excluded based on these data. Taken together these data show that several key antiapoptotic genes are expressed in CML CD34$^+$ cells with some also differentially expressed compared to the normal counterparts. Based on this result, it is possible to speculate that autocrine TNF-α would relay survival signals in this cell context thus providing a possible explanation for the biological effects seen on CML CD34$^+$ cells following TNF-α inh treatment and rescue with exogenous TNF-α .

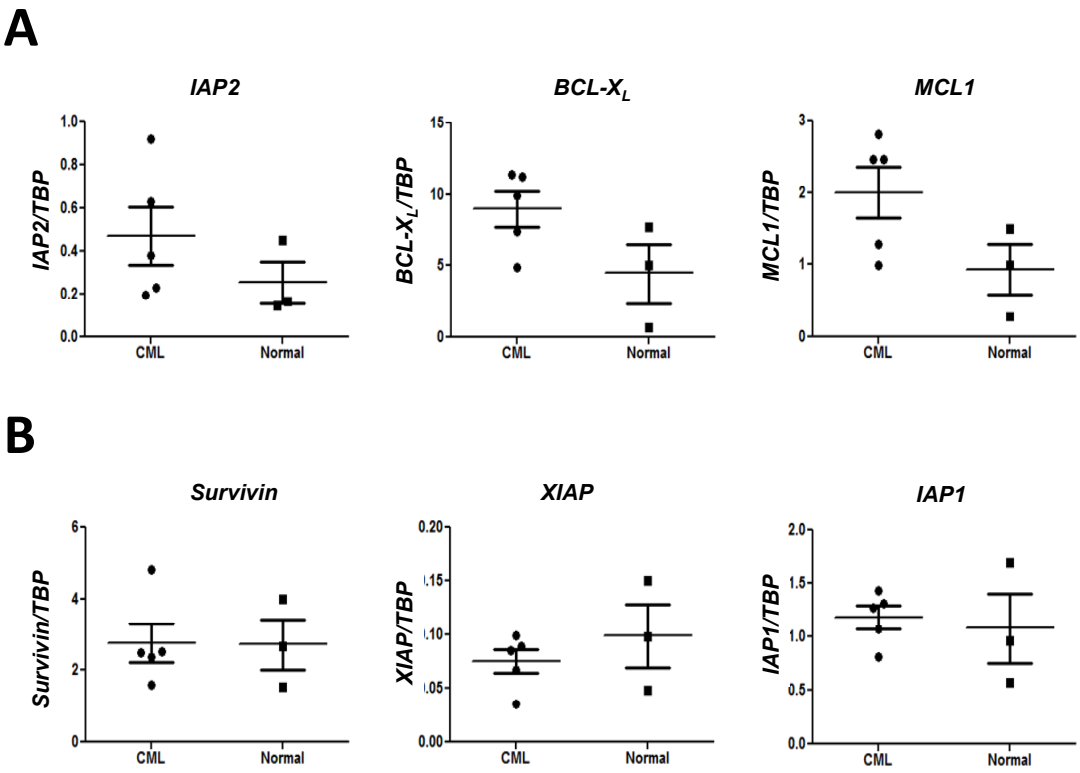


Figure 5-4 Quantitative mRNA expression of key antiapoptotic genes in CML and normal CD34$^+$ cells

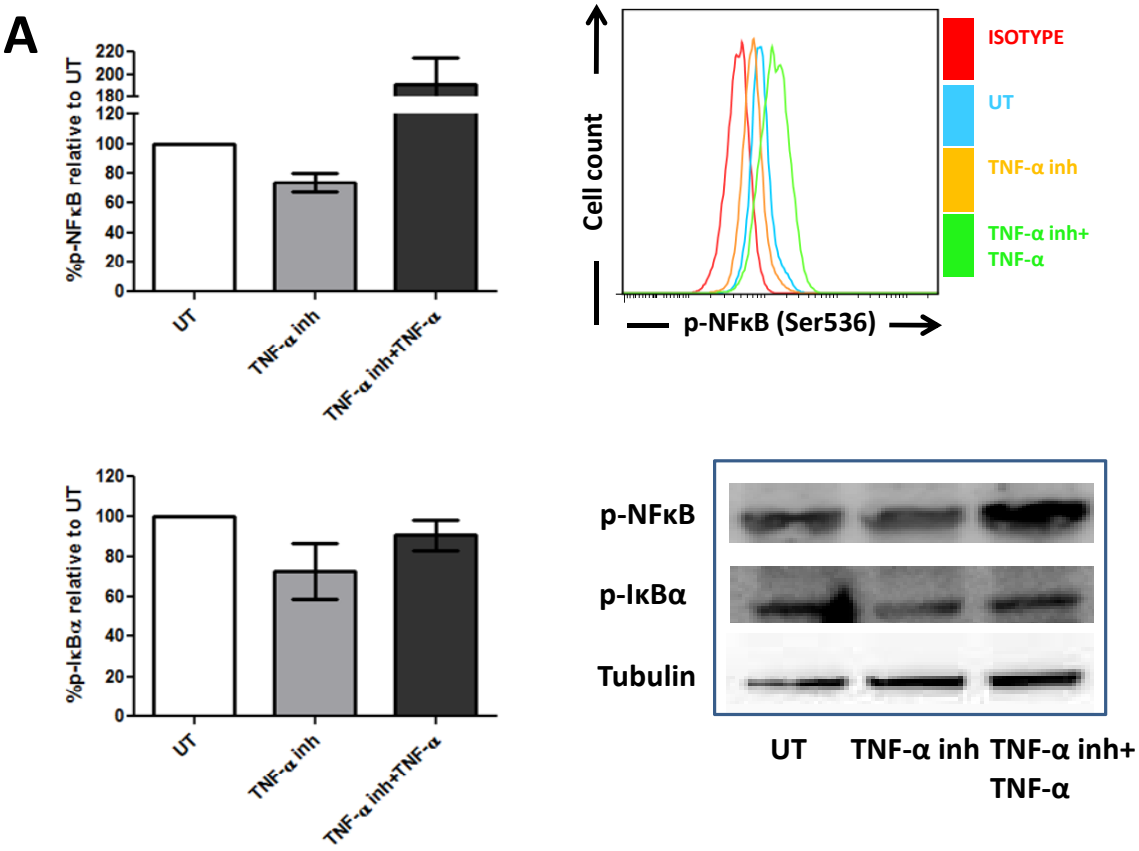
mRNA expression for each gene was measured in CML (n=5) and normal (n=3) CD34$^+$ samples following recovery and overnight culture in SFM+PGF and SFM+HiGF respectively. Levels of expression were calculated using the $2^{-\Delta Ct}$ method after normalisation of each gene mRNA expression levels against the expression levels of a housekeeping gene (*TBP*) within each sample. Panel A shows genes which appear to be differentially expressed between CML and normal CD34$^+$ cells, while panel B shows the ones for which no obvious difference in gene expression levels was detected.

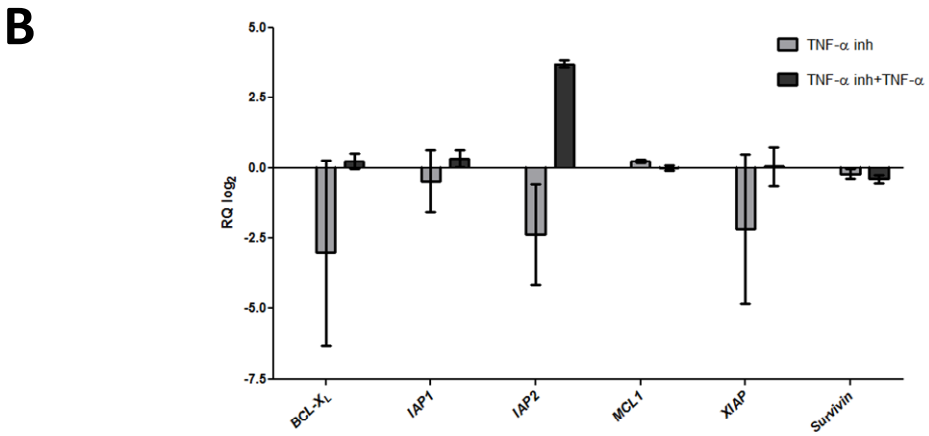
5.1.4.2 Analysis of the effects of TNF-α inhibition and stimulation on NFκB pathway in CML CD34$^+$ cells

TNF-α can transmit its proliferative signals through various pathways, including MAP kinase, activator protein-1 (AP-1) transcription factor and NFκB activation[134]. In order to investigate further the mechanisms behind the effects seen following TNF-α inhibition and stimulation in CML CD34$^+$ cells, the NFκB pathway was analysed in more detail by both protein and gene expression assays. The level of phosphorylation of NFκB on serine 536, a known activation site following TNF-α stimulation[152], was measured by intracellular flow cytometry and western blot analysis as a readout for activation of the pathway. Moreover the levels of phosphorylation on serine 32 and 36 of IκBα were also measured by the same techniques as further confirmation of the effects seen on the pathway. IκBα is a negative regulator of NFκB as its binding prevents nuclear translocation and activation of NFκB. However upon phosphorylation on the above mentioned serines (by the IKK complex following TNF-α stimulation), IκBα is tagged for proteasomal degradation which leads to the release of NFκB and its nuclear translocation and activation. Therefore in simple terms, high levels of phosphorylation of both NFκB and IκBα indicate an active pathway, while reduction in both proteins' phosphorylation levels reflects pathway inhibition[150].

TNF-α inh consistently reduced the level of phosphorylation of both NFκB and IκBα. However the magnitude of the effect was variable and overall limited to a non significant 20 to 25% reduction, suggesting that the NFκB pathway was kept active to a certain level even when autocrine TNF-α was inhibited, possibly via BCR-ABL. Exogenous TNF-α significantly increased the levels of p-NFκB ($p<0.001$) and p-IκBα, thus confirming that TNF-α in the context of CML CD34$^+$ cells causes activation of this pathway (figure 5-5 A). The effects of TNF-α inh were also assessed with regards to the levels of expression of NFκB pathway target genes, such as *BCL-X_L*, *IAPs*, *survivin* and *XIAP*. As shown in figure 5-5 B, TNF-α inh consistently reduced *IAP2* levels, while TNF-α increased them. For all other genes either no significant changes were observed or there was a high degree of patient sample variability, which precluded drawing any conclusion on the effects of the treatment on these gene expression levels. Finally to exclude that the effects seen with TNF-α inh were secondary to off-target inhibition of BCR-ABL kinase activity, the levels of two downstream targets of BCR-ABL, p-CrKL and p-STAT5, were measured following treatment with TNF-α inh. Neither p-CrKL nor p-STAT5 levels were reduced by TNF-α inh while NL, as expected, led to inhibition of both (figure 5-5 C).

Overall these data support the role of NFκB in TNF-α signalling in CML $CD34^+$ cells which helps to explain the effects seen in the cell biology assays. However given that NFκB is only partially inhibited by TNF-α inh, it is possible that other pathways, which have not been investigated, might also play a role in the effects seen. The reason behind the partial effects seen on NFκB activation with TNF-α inh are likely due to residual activation of this pathway by BCR-ABL which was not inhibited by the TNF-α inh. Although consistent changes at gene expression levels were seen only for *IAP2* amongst all NFκB target genes tested, it cannot be excluded that TNF-α inh might have regulated expression of these proteins at the post-translational level. Moreover amongst all NFκB target genes, *IAP2* appears to play a key role in redirecting TNF-α signals towards a prosurvival and proliferative outcome, because of its ability to further activate NFκB via a positive feedback loop[158,161]. In fact the clear activation of NFκB following TNF-α rescue experiments further supports the role of NFκB pathway in the signalling relayed by TNF-α in CML $CD34^+$ cells.


A
%p-NFκB relative to UT
220
200
180
120
100
80
60
40
20
0
UT
TNF-α inh
TNF-α inh+TNF-α
Cell count
p-NFκB (Ser536)
ISOTYPE
UT
TNF-α inh
TNF-α inh+ TNF-α
%p-IκBα relative to UT
120
100
80
60
40
20
0
UT
TNF-α inh
TNF-α inh+TNF-α
p-NFκB
p-IκBα
Tubulin
UT
TNF-α inh
TNF-α inh+ TNF-α


B
TNF-α inh
TNF-α inh+TNF-α
RQ log2
5.0
2.5
0.0
-2.5
-5.0
-7.5
BCL-XL
IAP1
IAP2
MCL1
XIAP
Survivin

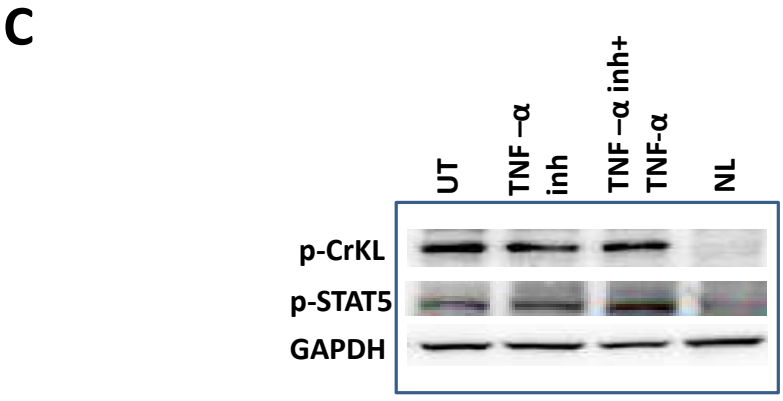

C
UT
TNF –α inh
TNF –α inh+ TNF-α
NL
p-CrKL
p-STAT5
GAPDH

Figure 5-5 Effects of TNF-α inhibition and stimulation on NFκB and BCR-ABL activity in CML CD34$^+$ cells

p-NFκB and p-IκBα levels were measured by intracellular flow cytometry (A, left panels) in 3 CML CD34+ samples after 24 hours of incubation in suspension cultures with TNF-α inh or TNF-α inh+TNF-α. Levels of phosphorylation of both proteins were calculated as described in 2.3.2.1. Representative plot for changes in p-NFκB is shown in panel A, top right. Representative western blot analysis for changes in the levels of the same proteins is shown in panel A, bottom right. Tubulin was used as a loading control. Gene expression changes for NFκB target genes was measured in 3 CML samples following 24 hours culture in SFM+PGF with TNF-α inh or TNF-α inh plus TNF-α. Differences in gene expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator) (B). P-CrKL and p-STAT5 levels were measured by western blot in CML CD34$^+$ cells (n=3) treated for 24 hours with TNF-α inh, TNF-α inh+TNF-α or NL. Representative blot of 3 is shown. GAPDH was used as a loading control (C).

5.1.4.3 Analysis of the effects of TNF-α inhibition and stimulation on GFs and CSF2RB expression in CML CD34$^+$ cells

TNF-α has been shown to upregulate expression of several GFs and cognate receptors in different model systems including human and murine HSPC[145,146,136,147,412]. This feature could also be secondary to NFκB activation, given its prominent role in regulating GFs production by immune cells, although this link has not been proven following stimulation of haemopoietic and stromal cells by TNF-α. Given the proliferative effects caused by TNF-α in CML CD34$^+$ cells, it was interesting to investigate if the expression of any GFs and receptors were indeed regulated by TNF-α in this cell context too. Given their differential expression in CML SPC versus normal HSPC (see 3.1.3), mRNA levels for *IL-4*, *GM-CSF* and *CSF2RB* levels were measured following 24 hour treatment with TNF-α inh or TNF-α inh plus TNF-α in CML CD34$^+$ cells. CSF2RB protein levels were also measured following 48 hours in the same treatment conditions. *GM-CSF* was not detected in the three UT CML CD34$^+$ samples (consistent with the fact that autocrine production of this GF is not always detected in CML SPC, see 3.1.2 and 3.1.3) and following TNF-α inh treatment, but became detectable following TNF-α inh plus TNF-α treatment (figure 5-6 A). Instead, for both *IL-4* (detected in 2 out of 3 samples) and *CSF2RB* (detected in all 3 samples), TNF-α inh treatment caused a variable degree of reduction in their level of expression compared to UT, which was rescued with addition of exogenous TNF-α. Interestingly a similar pattern was also observed for *SCF* (detected in 2 out of 3 samples), a GF the expression of which was observed in CML CD34$^+$ cells, although at a level not different from normal CD34$^+$ cells (not shown) (figure 5-6 B). Moreover consistent with gene expression findings, CSF2RB protein levels in CML CD34$^+$ cells were also respectively downregulated and upregulated following TNF-α inh and TNF-α inh plus TNF-α treatment (both $p<0.05$) (figure 5-6 C).

Overall these data show that modulation of TNF-α activity in CML CD34$^+$ cells can affect autocrine production of proliferative GFs expressed by CML CD34$^+$ cells. These correlative changes may provide another potential mechanism by which autocrine TNF-α can act as a proliferative and survival cue in CML CD34$^+$ cells.

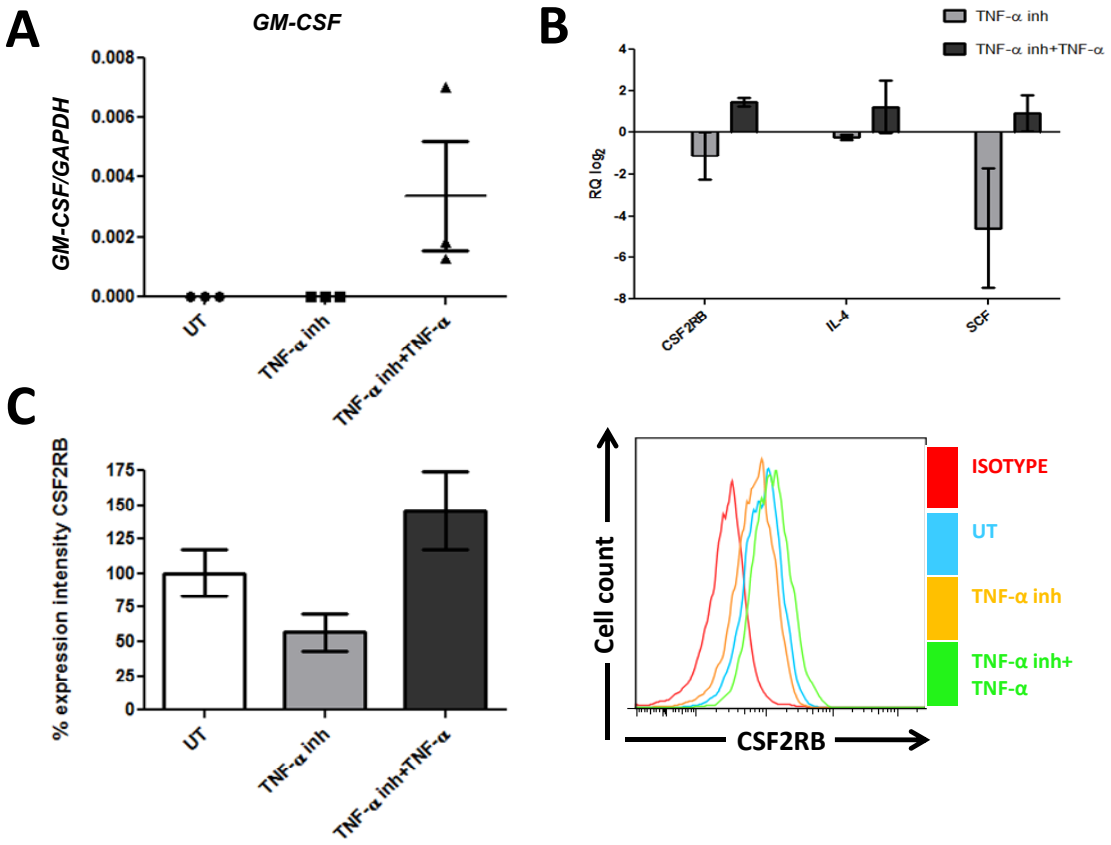


Figure 5-6 Effects of TNF-α inhibition and stimulation on GFs and CSF2RB expression by CML CD34$^+$ cells

Gene expression changes for candidate GFs were measured in 3 CML samples following 24 hours culture in SFM+PGF with TNF-α inh or TNF-α inh plus TNF-α. Levels of expression for *GM-CSF* were calculated using the $2^{-\Delta Ct}$ method after normalisation within each sample of GFs mRNA expression levels against the expression levels of a housekeeping gene (*GAPDH*). Levels of expression for UT and TNF-α inh were set at 0 as they were undetected (A). Differences in gene expression levels following treatment for *CSF2RB*, *IL-4* and *SCF* were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator) (B). Levels of protein expression of CSF2RB were measured by flow cytometry analysis and expressed as MFI of the antibody stained cells over MFI of cells stained with a matched isotype antibody. The average expression levels in the TNF-α inh and TNF-α inh plus TNF-α treated cells was then plotted as percentage of the average expression levels in UT cells (C, left panel). Representative plots of CSF2RB protein expression (C, right panel).

5.1.5 Assessment of the effects of TNF-α inhibition in combination with NL on CML CD34$^+$ cells survival and proliferation

Following demonstration that autocrine TNF-α supports survival and proliferation of CML CD34$^+$ cells, the next step was to assess if its effects were present even when BCR-ABL kinase was inhibited by a TKI. The lack of significant modulation of TNF-α production by CML CD34$^+$ cells following NL treatment (see 3.1.4 and 3.2.2) would suggest that TNF-α effects might indeed be BCR-ABL kinase independent. In order to prove this, 5μM NL was therefore combined with TNF-α inh at a fixed concentration (3μM) and effects on CML CD34$^+$ cell survival and proliferation measured. The combination treatment was able to induce higher levels of apoptosis at both 48 ($p<0.05$ relative to NL and $p<0.01$ relative to TNF-α inh) and 72 hours ($p<0.01$ relative to both NL and TNF-α inh) compared to either single agent alone (figure 5-7 A). Total CFC output was also further reduced by combined treatment and this was mainly due to a reduction in the more primitive CFU-GM compared to both NL and TNF-α inh single agent treatment (figure 5-7 B). Profound effects on both proliferative and quiescent CML CD34$^+$ cells, when assessed by CFSE staining, were also observed. Besides a generalised effect on reducing proliferation of CML CD34$^+$ cells, the combination treatment caused a significant reduction in CD34$^+$ cells recovered in both the undivided population and within the early divisions relative to UT ($p<0.05$ for the undivided and $p<0.001$ for divisions 1, 2 and 3). Moreover a further overall reduction in the total recovered CD34$^+$ cells compared to both single agents was noted (figure 5-7 C). The effects seen on CFSE recovery combined with the observation that levels of autocrine TNF-α production are similar in both CML CD34$^+$ CD38$^+$ and CD34$^+$ CD38$^-$ cells (see 3.1.3) suggest that TNF-α could play a relevant role in the survival of a more primitive stem cell population. In order to test this hypothesis, apoptosis induction was measured specifically in the CFSE^{max} population (which reflects a more quiescent and primitive stem cell subset) and in a CML CD34$^+$ CD38$^-$ sorted population (a more primitive stem cell subset based on cell surface markers). In both cases the combination of TNF-α inh and NL induced significantly higher levels of apoptosis compared to either single agents alone ($p<0.05$ for both comparisons in both experiments) (figure 5-7 D). Overall these data suggest that combining TNF-α inhibition with TKI reduces CML SPC survival and inhibits their proliferation more than either approach on its own. Moreover combination treatment is also able to target more efficiently a primitive stem cell subset which is notoriously resistant to standard TKI therapy.

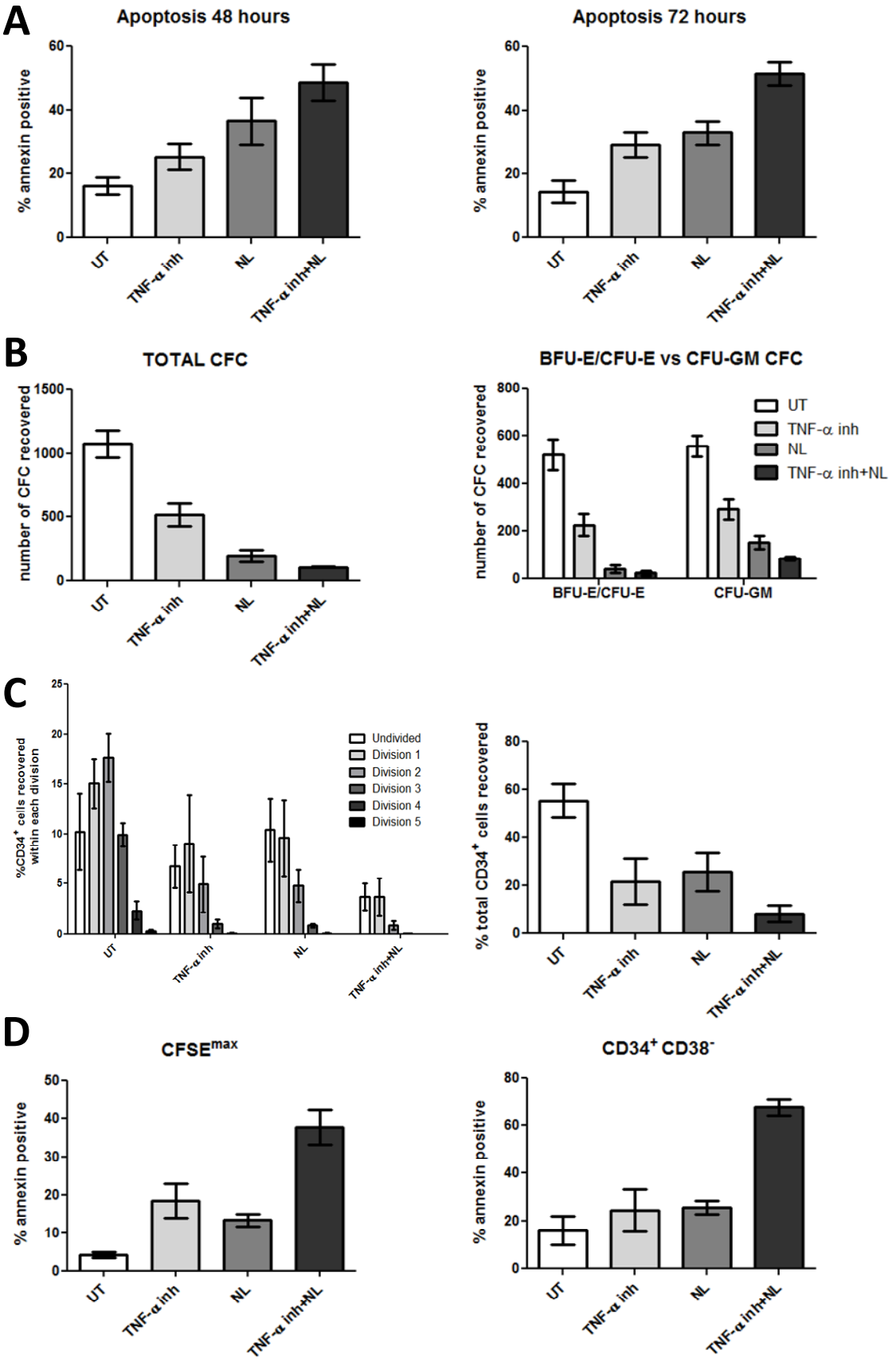

A
Apoptosis 48 hours
% annexin positive
60
40
20
0
UT
TNF-α inh
NL
TNF-α inh+NL
Apoptosis 72 hours
% annexin positive
60
40
20
0
UT
TNF-α inh
NL
TNF-α inh+NL
B
TOTAL CFC
number of CFC recovered
1500
1000
500
0
UT
TNF-α inh
NL
TNF-α inh+NL
BFU-E/CFU-E vs CFU-GM CFC
number of CFC recovered
800
600
400
200
0
UT
TNF-α inh
NL
TNF-α inh+NL
BFU-E/CFU-E
CFU-GM
C
%CD34+ cells recovered within each division
25
20
15
10
5
0
Undivided
Division 1
Division 2
Division 3
Division 4
Division 5
UT
TNF-α inh
NL
TNF-α inh+NL
% total CD34+ cells recovered
80
60
40
20
0
UT
TNF-α inh
NL
TNF-α inh+NL
D
CFSEmax
% annexin positive
50
40
30
20
10
0
UT
TNF-α inh
NL
TNF-α inh+NL
CD34+ CD38-
% annexin positive
80
60
40
20
0
UT
TNF-α inh
NL
TNF-α inh+NL

Figure 5-7 Effects of TNF-α inhibition combined with NL on survival and proliferation of CML SPC

CML $CD34^+$ cells (n=5) were cultured in SFM+PGF with TNF-α inh, NL or their combination. Apoptosis was measured by annexin-V/7AAD staining at 48 and 72 hours (A). At 72 hours, 3,000 cells from each of the arms were plated in standard CFC. Number of colonies recovered in total and based on their morphology from each treatment arm are plotted (n=3) (B). CML $CD34^+$ cells (n=4) were CFSE stained and then cultured for 72 hours with TNF-α inh, NL or their combination. Percentage of starting $CD34^+$ cells recovered within each division and in total following treatment were calculated as explained in 2.3.2.3 (C). Apoptosis was measured by annexin-V/7AAD following 72 hours treatment in the same conditions within the $CFSE^{max}$ population (n=5) and in previously sorted $CD34^+$ $CD38^-$ cells (n=2) (D).

5.1.6 Investigation of the mechanisms underlying the effects of TNF-α inhibition in combination with NL on CML CD34$^+$ cells survival and proliferation

In order to clarify the mechanisms underlying the effects seen when combining TNF-α inh with NL in CML CD34$^+$ cells, levels of p-NFκB and p-IκBα were first measured by intracellular flow cytometry. The combination treatment caused a further 20% reduction in the phosphorylation levels of both proteins ($p<0.05$ for p-NFκB and not significant for p-IκBα), suggesting that the effects seen are at least partially secondary to a stronger inhibition of the NFκB pathway with the combined treatment (figure 5-8 A). However it should be noted that levels of activation of both proteins were still about 50% following combination treatment.

Gene expression changes were also measured for the same antiapoptotic genes previously assessed when investigating the mechanisms of action of TNF-α inhibition. Interestingly, except for *survivin* where the combination treatment caused a reduction previously not present with TNF-α inh alone, for all the others the addition of NL either did not cause any change in the levels (*BCL-X_L*, *IAP1*, *MCL1* and *XIAP*) or even reverted the changes seen with TNF-α inh on its own (*IAP2*), thus suggesting that complex feedbacks are generated when combining the two approaches which result in a more global modification of the signalling output within the cell than simple cumulative effects of the two drugs on the same pathway (figure 5-8 B). In fact the combination treatment appeared to determine effects on other key genes which were not individually targeted by either agent alone and which are overexpressed in CML versus normal CD34$^+$ cells, such as *MYC* (figure 5-8 C).

Taken together these data show that the mechanism of action of TNF-α inh with NL on CML CD34$^+$ cells are complex and not easily explained by an additive effect on a single pathway such as NFκB.

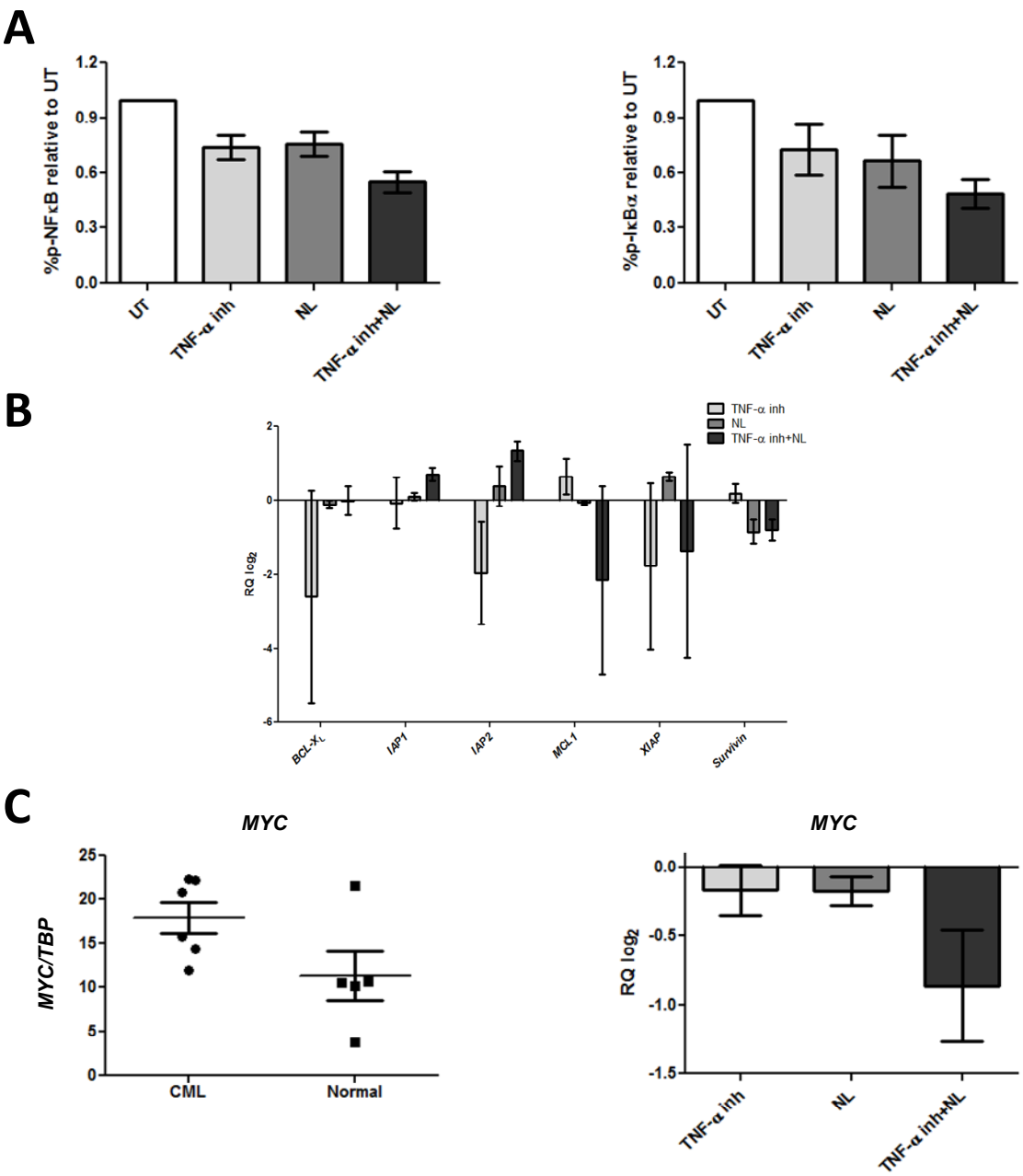


Figure 5-8 Effects of TNF-α inhibition in combination with NL on NFκB pathway and *MYC* in CML CD34$^+$ cells

p-NFκB and p-IκBα levels were measured by intracellular flow cytometry in 3 CML CD34$^+$ samples after 24 hours of incubation in suspension cultures with TNF-α inh, NL or their combination. Levels of phosphorylation of both proteins were calculated as described in 2.3.2.1 (A). Gene expression changes for NFκB target genes and *MYC* were measured in 3 CML samples following 24 hours culture in the same conditions. Differences in gene expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator) (B and C, right panel). mRNA expression for *MYC* was measured in CML (n=6) and normal (n=5) CD34$^+$ samples following recovery and overnight culture in SFM+PGF and SFM+HiGF respectively. Levels of expression were calculated using the $2^{-\Delta Ct}$ method after normalisation of *MYC* mRNA expression levels against the expression levels of a housekeeping gene (*TBP*) within each sample (panel C, left).

5.2 Investigation of the role of autocrine TNF-α in CML CD34$^+$ cells using a TNF-α neutralising antibody

Classically autocrine growth has been thought to be secondary to stimulation of a cell by its own secreted GFs via their binding to their cognate membrane receptors[413]. Therefore in order to confirm our findings using a TNF-α small molecule inhibitor by a different approach, CML CD34$^+$ cells were cultured in the presence of a TNF-α neutralising antibody or a matched isotype control antibody. Interestingly the TNF-α neutralising antibody only mildly increased levels of apoptosis in CML CD34$^+$ cells compared to isotype antibody and as a result no changes in apoptosis levels were observed when exogenous TNF-α was added to the culture (figure 5-9 A). The effects of the TNF-α neutralising antibody on p-NFκB levels were also measured. The TNF-α neutralising antibody again did not change levels of p-NFκB relative to isotype. Notably addition of exogenous TNF-α increased the levels of p-NFκB in the presence of isotype antibody but not in the presence of TNF-α neutralising antibody, which confirms the specificity of the TNF-α neutralising antibody used (figure 5-9 B). These results suggest that the previously demonstrated autocrine effects of TNF-α in CML CD34$^+$ cells are not due to the secreted form of TNF-α binding to its cognate surface receptor.

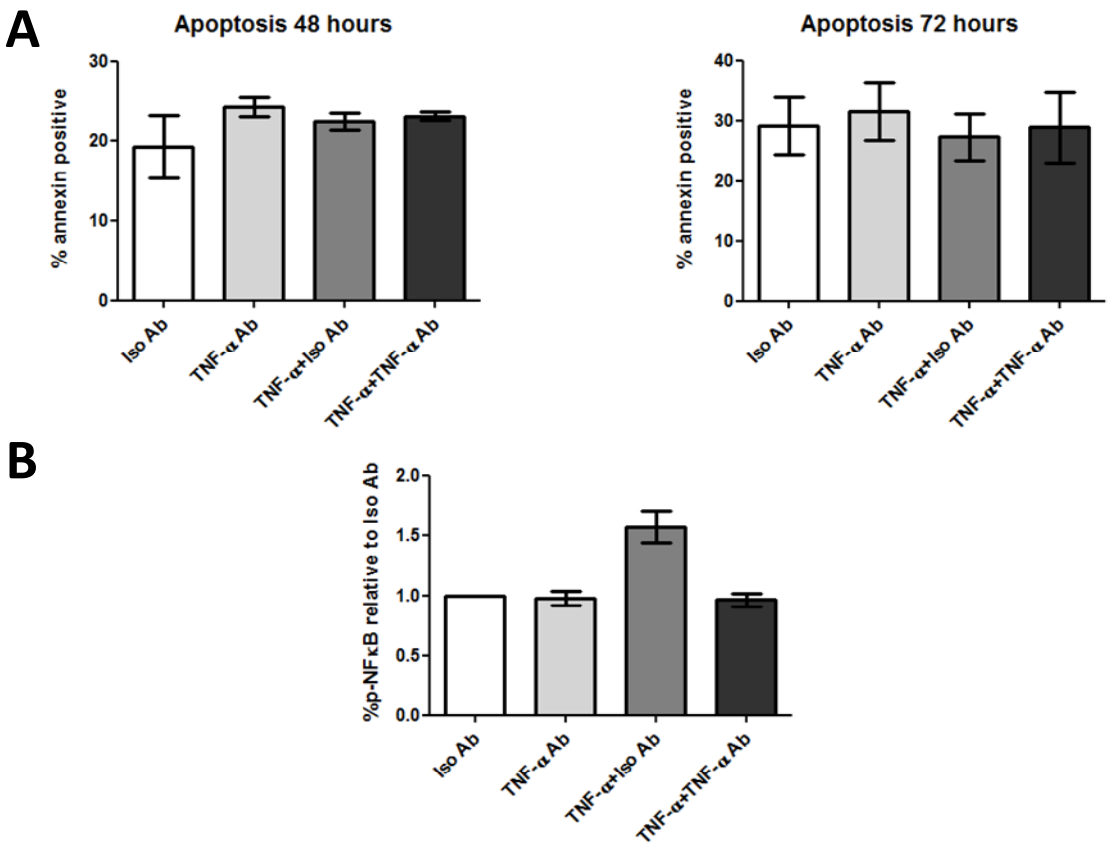


Figure 5-9 Effects of TNF-α neutralising antibody in CML CD34$^+$ cells
CML CD34$^+$ cells (n=3) were cultured in SFM+PGF with TNF-α neutralising antibody (TNF-α Ab) or matched isotype control antibody (Iso Ab) both at 25μg/mL in the presence or absence of exogenous TNF-α at 1ng/mL. Apoptosis was measured by annexin-V/7AAD staining at 48 and 72 hours (A). p-NFκB levels were measured by intracellular flow cytometry in 3 CML CD34$^+$ samples after 24 hours of incubation in the same conditions. Levels of phosphorylation of the protein were calculated as described in 2.3.2.1 and expressed as a percentage of Iso Ab treated cells (B).

5.3 Summary and future directions

The work presented in this chapter shows that inhibition of autocrine TNF-α signalling using a specific small molecule TNF-α inhibitor is able to induce apoptosis and reduce proliferation in CML $CD34^+$ cells. TNF-α inhibition also appears to be particularly effective in targeting the $CFSE^{max}$ population as levels of apoptosis within this population were higher on exposure to the small molecule inhibitor. Given that the amount of autocrine TNF-α produced by CML $CD34^+$ $CD38^-$ LSC was similar to that produced by more mature CML LPC ($CD34^+$ $CD38^+$) (see 3.1.3) this last result is not surprising. The evidence supporting this conclusion was gathered mainly by using a small molecule TNF-α inhibitor with rescue experiments using exogenous TNF-α to confirm the specificity of the effects seen. It was therefore initially surprising that when a TNF-α neutralising antibody was used to further confirm the findings observed with the TNF-α inh, the same effects on survival of CML $CD34^+$ cells were not seen. However it should be noted that autocrine stimulation can occur by different mechanisms: autocrine GFs may be secreted and bound to their surface receptors or may act by binding to an internal cognate receptor as shown already in transformed cells and haemopoietic cells[414,415]. The latter mechanism therefore appears to play a major role in the autocrine effects of TNF-α in CML $CD34^+$ cells when taking into account the results obtained with both TNF-α inh and TNF-α neutralising antibody. It is interesting to note also that the internal binding mechanism for autocrine stimulation has already been described in other BCR-ABL positive cells capable of autocrine GFs production[269,361] which might suggest this is a common mechanism used in cells transformed by BCR-ABL. Moreover protein expression of TNF-α by CML $CD34^+$ cells was only detected using intracellular flow cytometry analysis (see 3.2.1), but not when other techniques specifically aimed to detect secreted proteins (such as ELISA) were employed (not shown).

However other possible explanations including technical ones should also be considered. TNF-α is produced as both a soluble protein and a membrane bound protein[134] and although the TNF-α antibody used was specifically tested for neutralising purposes, it was not epitope mapped. It is therefore possible that the epitope recognised could be part of the transmembrane or intracytoplasmic structure of TNF-α. If that is the case the antibody might not be able to bind TNF-α when expressed in its transmembrane form thus explaining its reduced efficacy compared to the TNF-α small molecule inhibitor which instead acts by disrupting the trimeric structure of active TNF-α[410]. It is also possible that the small molecule inhibitor was more potent than the neutralising antibody although this

is unlikely as the neutralising antibody was used at high saturating concentrations. Alternatively the small molecule inhibitor effects might be not specific. Against this latter hypothesis however are several observations:

1) Although to different extents, the TNF-α inh effects were consistently rescued by exogenous TNF-α. It is possible that if intracellular receptor activation is indeed present a significant proportion of the TNF-α receptors might be occupied before reaching the cell surface which might also explain why the rescue experiments with exogenous TNF-α were not always able to fully revert the effects caused by the TNF-α inh.

2) TNF-α inh showed no effects on normal $CD34^+$ cells (which were shown to produce only low levels of TNF-α) which again supports its specificity (it is worth noting that this finding would also support the theory that a truly autocrine effect in *in vitro* culture can only be seen when levels of autocrine produced GFs are sufficient and can help to explain also the lack of efficacy of JAK2 inhibitors on CML cells in the absence of exogenous GFs described in the previous chapter).

3) No effects of TNF-α small molecule inhibitor on BCR-ABL kinase activity were demonstrated which excludes therefore the possibility of off-target effects of this compound at least on BCR-ABL kinase.

A potential way to clarify this further would be to silence the expression of TNF-α in CML $CD34^+$ cells by RNA interference techniques to see if the findings obtained with TNF-α inh are confirmed. This is currently in planning and will help clarify this issue.

Therefore based on the above considerations, it is reasonable to assume that the effects seen with the TNF-α inh were secondary to inhibition of autocrine intracellular TNF-α. The finding that autocrine TNF-α acts as a survival cue in CML SPC is not surprising given that this has already been reported in other cancer systems and TNF-α itself can effectively relay stimulatory signals depending on the cell context[396,409]. The importance of the cell context for the effects of TNF-α has been long known. It has been shown that TNF-α is able to induce apoptosis only in cells where the NFκB pathway is inactive[164]. NFκB activation is necessary to allow transformation of murine BM by BCR-ABL[253] and therefore must be active in primitive CML cells. Our results from gene expression analysis support this as they have shown that CML $CD34^+$ cells express high levels of several antiapoptotic genes, in particular of the *IAP* family, which are known to be under direct

control of NFκB and can also activate it further with a positive feedback loop. The IAP1 and IAP2 proteins have been shown to play a central role in redirecting TNF-α signalling from an apoptotic to a prosurvival outcome[161] thus providing the right cell context in CML $CD34^+$ cells for autocrine TNF-α to act as a prosurvival cue. It was interesting to see that normal $CD34^+$ cells, which produce significantly less autocrine TNF-α than their CML counterparts and also express reduced levels of several antiapoptotic proteins, do not appear to rely on this autocrine loop as TNF-α inhibition did not cause any effects on their survival. This would also suggest that a therapeutic window could be present if a strategy targeting TNF-α signalling should be developed for treating CML.

The putative role of TNF-α as a survival cue in CML $CD34^+$ cells would also be consistent with recent observations published by Zhang *et al* which have shown, using a transgenic CML mouse model, that TNF-α is produced at higher level in the BM of CML mice relative to wild type. Moreover they also showed higher levels of *TNF-α* mRNA expression in the BM MNC from CML patients compared to normal controls. In functional studies, they also showed that mouse CML LSC had a growth advantage and were protected from apoptosis compared to normal control stem cells when cultured in the presence of physiological concentrations of TNF-α[276]. Conversely reports published several years ago suggested a different role for TNF-α in CML as exposure to TNF-α caused CML cell death. However it is worth noting that in none of these reports purified populations of CML SPC were used, but instead a MNC fraction with only a small proportion of $CD34^+$ cells. Moreover in some cases very high concentrations of TNF-α (up to 100ng/mL) were used[405,408]. As the effects of TNF-α are highly concentration and cell context dependent, as also shown in normal haemopoiesis where its proliferative effects on progenitor cells are counteracted by negative/inhibitory effects on more differentiated cells[140], these considerations might help to explain the discrepancy between our results and those published in these early reports. It is intriguing to speculate that different levels of activation of NFκB underlie the different effects observed when more primitive or mature cell subsets are exposed to TNF-α.

The mechanisms underlying the effects of autocrine TNF-α inhibition were also investigated. As TNF-α is known to activate NFκB to relay its survival signals, the effects of TNF-α inhibition on this pathway were mainly assessed. Reduced activation of this pathway following TNF-α inh treatment was demonstrated using different readouts, including changes in phosphorylation levels of two key proteins within the pathway (NFκB itself and IκBα) and gene expression changes in key NFκB targets. The effects however on

NFκB and IκBα phosphorylation were not dramatic and amongst all the NFκB target genes tested only consistent downregulation of *IAP2* was observed following TNF-α inh. These results would suggest that beside NFκB, TNF-α inh might also affect other pathways which were not investigated in the current work. TNF-α is indeed known to act on several pathways and transcription factors, such as MAP kinase and AP-1[134]. However, TNF-α rescue experiments led to activation of the NFκB pathway showing that TNF-α does signal through this pathway, which is therefore at least partly contributing to the effects seen. It is also important to consider that BCR-ABL induced NFκB activation was still present in cells treated with TNF-α inhibitor on its own (as previously stated TNF-α inh did not have any effects on BCR-ABL kinase activity) which might explain why the inhibition of this pathway was only partial.

TNF-α is also known to regulate the production of several GFs by other cell types (see 1.1.2.3). It was interesting therefore to see if autocrine TNF-α might contribute to autocrine production of GFs and cognate receptors in CML $CD34^+$ cells too. Interestingly it was found that production of several GFs (*IL-4*, *GM-CSF* and *SCF*) was indeed reduced upon TNF-α inh treatment and stimulated by exogenous TNF-α. In particular the CSF2RB receptor, which had previously been shown to be upregulated in CML SPC compared to normal counterparts, was shown to be respectively down and upregulated upon TNF-α inhibition and stimulation, both at the protein and gene expression level. This result is in keeping with similar results shown in normal haemopoietic cells where TNF-α was also able to upregulate the expression levels of CSF2RB[147]. This might explain some of the proliferative effects caused by TNF-α and might also provide a possible link between TNF-α and JAK2/STAT5 axis. It is noticeable that both inhibition of JAK2 and TNF-α have similar effects on the survival and proliferation of CML cells. Although the consequences of TNF-α inhibition or stimulation on STAT5 activity were not formally assessed, hints towards upregulation of STAT5 activity upon TNF-α treatment were provided by the gene expression data showing upregulation of the expression of genes such as *BCL-X_L* (figure 5-5 B) and *cyclin D1* (data not shown in the thesis) which are also known to be STAT5 target genes. This aspect of TNF-α signalling however would require further investigation to be confirmed and clarified.

The effects of TNF-α inhibition in conjunction with BCR-ABL inhibition were also assessed. This was done because TNF-α autocrine production had been shown to be mainly BCR-ABL kinase independent and also because TNF-α inh did not show any off-target effects on BCR-ABL kinase activity. Interestingly this combination treatment showed a

dramatic effect on induction of apoptosis in CML $CD34^+$ cells, while also markedly reducing their proliferation and colony forming capacity. Moreover the combination was particularly effective in killing the most primitive CML LSCs defined based on their quiescent phenotype or surface markers. A significant reduction in the $CD34^+$ cells recovered in the undivided gate was caused by the combination treatment relative to UT. This was coupled with a significant induction of apoptosis in the $CFSE^{max}$ population and in sorted leukaemic $CD34^+$ $CD38^-$ cells following the combination treatment and relative to UT and either single agent alone. These observations suggest that, despite the presence of TKI, TNF-α is still able to relay survival signals which become even more important in this context, supporting a synthetic lethality model for the effects seen similar to the one described when combining JAK2 inhibitors and TKI. These observations would therefore give support to a therapeutic strategy combining TNF-α inhibition with TKI.

It was interesting to note that the presence of TKI did not alter the cell context of CML $CD34^+$ cells to the extent of rendering autocrine TNF-α signalling proapoptotic. In fact when levels of several key antiapoptotic genes were measured following NL treatment in CML cells no significant changes in their levels were noted which suggest that, even following TKI therapy, autocrine TNF-α would still act in a cell context that favours its prosurvival signals. This observation also provides an explanation for the effects seen with combined treatment. However when the exact mechanisms of the combination treatment were investigated, in particular looking at the NFκB pathway, it appeared clear that the effects on signalling underlying the biological responses seen were more complicated than just a summation of effects on a particular pathway (NFκB in this case). As a possible explanation, it has already been shown that the effects of small molecule inhibitors on complex cellular systems are a consequence of all their on- and off-target actions and that, as a result of crosstalk at various levels, the complexity of their effects is enhanced when two drugs are applied simultaneously[384]. It is therefore also not surprising that novel points of convergence of the effects of two drugs applied simultaneously on a complex cell system can be identified as new targets explaining the effects seen following combination treatment. Interestingly the results were more suggestive of a novel and still not fully clarified mechanism behind the effects seen with combination treatment, with gene expression changes pointing towards *MYC* being a possible target of the combined treatment. MYC is a key transcription factor already shown to be essential for BCR-ABL mediated transformation[247] and also shown to play a key role in numerous other malignancies, including haemopoietic ones[416,417].

In conclusion the results shown in this chapter support further investigation of the role of autocrine TNF-α in CML biology, including potentially developing a strategy to target it therapeutically, in particular in combination with TKI. Although TNF-α inhibitory therapy is already available for several autoimmune disorders, based on the lack of efficacy of TNF-α neutralising antibody, a strategy using small molecule TNF-α inhibitors, such as the one used in this study, should be pursued in CML as this would also have the advantage to be orally administered. The one used in this chapter was originally described by a group working for the biopharmaceutical company Sunesis[410], however it does not appear to have been taken further in its clinical development. If *in vivo* experiments in animal models will prove supportive of the current findings and demonstrate potential lack of toxicity of this approach, it is therefore advisable that similar compounds be developed to assess if such a therapeutic strategy is really possible. Alternatively, small molecules interfering with TNF-α signalling, such as NFκB pathway inhibitors (such as IKK inhibitors) or IAP inhibitors (such as SMAC mimetics) could also be employed. This would take advantage, in an indirect way, of the autocrine secretion of TNF-α by CML cells (figure 5-10) by redirecting its signals towards an apoptotic outcome, as shown in other cancer systems. Both types of compounds are currently being clinically developed and our laboratory is already primed to test their efficacy in the CML field.

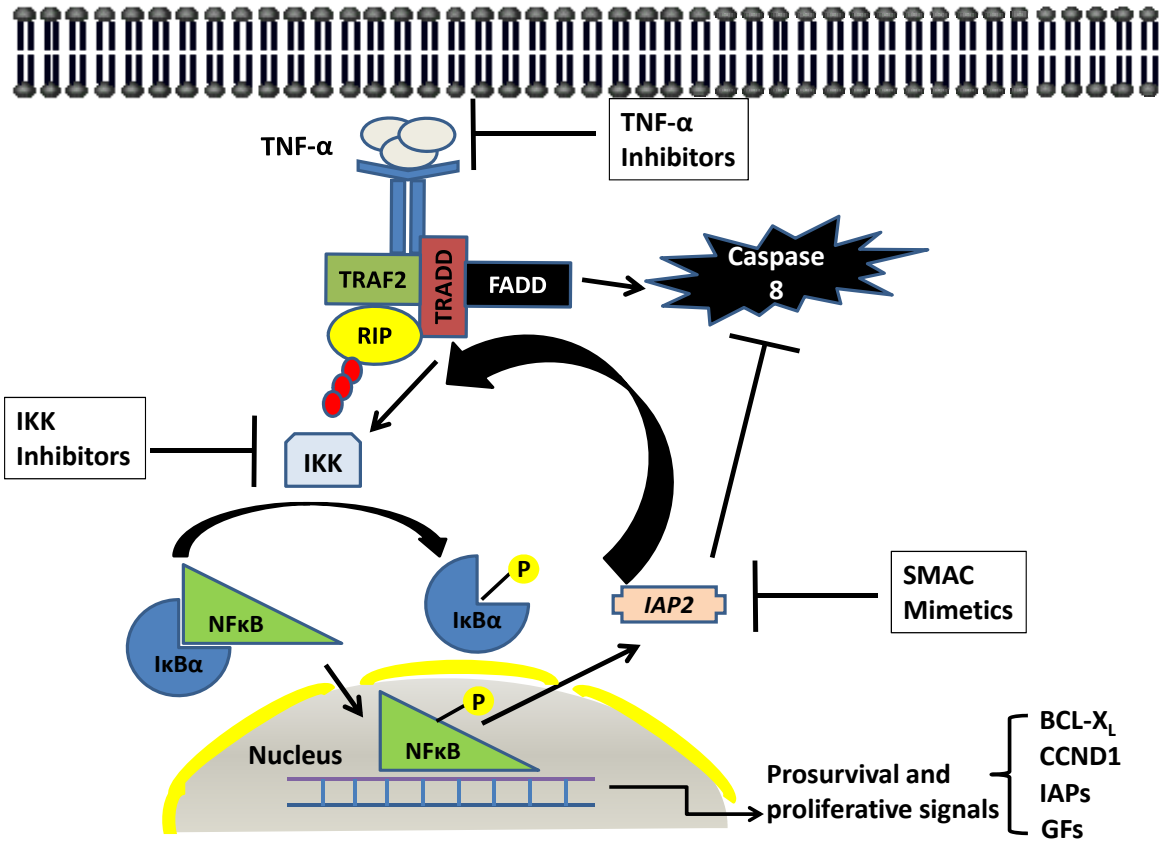


Figure 5-10 Possible strategies to interfere with autocrine TNF-α signalling in CML
TNF-α in CML cells activates the NFκB pathway leading to inhibition of its proapoptotic effects mainly by induction of IAP2 which blocks caspase activation. TNF-α inhibitors can interfere with this pathway. Other possible speculative approaches are inhibition of IKK and IAP2 through IKK inhibitors and SMAC mimetics. Both strategies would lead to reduction of NFκB activity and swing TNF-α signalling from prosurvival to proapoptotic as activation of caspases would not be counteracted anymore.

6 Results (IV) Investigation of putative autocrine and paracrine regulators of CML LSC quiescence and self-renewal

A defining characteristic of HSC is quiescence. This feature appears to be of critical biological importance in preventing stem cell exhaustion, particularly under conditions of stress, and ensuring maintenance of a self-renewing pluripotent cell population able to repopulate the entire BM[25,28] (note the term maintenance is used here to signify preservation of self-renewal potential). Therefore quiescence and maintenance are two closely linked attributes of HSC. Deletion of genes central to HSC quiescence leads to their depletion and exhaustion as demonstrated for example in mice carrying a deletion of cell cycle negative regulators, such as the CKIs *Cdkn1a/p21*[95] and *Cdkn1c/p57*[96]. Several extrinsic regulators of normal HSC quiescence/maintenance have been described, including TGF-β1[27,97], TPO[98,99], Wnt/β-catenin[100-102], Hh[103,104], integrin[105] and chemokine signalling[27,106,107] (see section 1.1.2.2 for a more detailed discussion).

Similarly to normal haemopoiesis, a quiescent LSC population has been demonstrated in CML, despite the highly proliferative phenotype induced by the BCR-ABL oncoprotein, and still detectable even in the presence of GFs stimulation[192]. Moreover LSC quiescence has been shown to be a resistance mechanism in the CP of disease following TKI therapy[328]. An improved understanding of the mechanisms controlling quiescence in LSC would therefore provide potential novel therapeutic targets for their eradication in CP CML and would advance our knowledge of LSC biology in general. Similarly to normal haemopoiesis, CML LSC quiescence and self-renewal appears also to be controlled by both extrinsic and intrinsic regulators. Amongst the extrinsic regulators Hh[104,265], Wnt/β-catenin[266-268] andTGF-β1, via its downstream transcription factors such as FOXO[111] and BCL6[378], are the most relevant. It is worth noting that a great degree of overlap exists between the pathways shown to play a role in both normal and CML stem cell maintenance. This therefore suggests that any strategy aiming to target such pathways in CML therapy has to rely on either increased expression of these regulators or higher dependency on these pathways in CML stem cells compared to their normal counterparts.

Recently our laboratory, in collaboration with Dr David Vetrie's laboratory, has used a combined approach including genome-wide expression and epigenetic analysis of normal

HSC ($CD34^+$ $CD38^-$) and HPC ($CD34^+$ $CD38^+$) and their leukaemic counterparts, to obtain a global view of the cellular processes that regulate stem cell maintenance in HSC and LSC (Dr K. Korfi PhD thesis, University of Glasgow, 2012). When looking at the gene expression changes in the transition from a normal HSC to a normal HPC, pathways potentially involved in HSC maintenance showed significant ($p<0.05$) downregulation, whereas pathways involved in proliferation were significantly upregulated in line with their known biological differences (see section 1.1) and previous gene profile studies[418]. However interestingly when comparing CML LSC to normal HSC, it appeared that despite having increased activity in pathways involved in cell division and proliferation, the expression levels of the pathways potentially involved in stem cell maintenance remained significantly unchanged. These pathways included Wnt, TGF-β signalling and chemokine signalling, as well as novel signalling pathways involving neurotransmitters (figure 6-1).

Moreover by examining genome-wide histone modification patterns using chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq), it was demonstrated that the majority of the putative HSC maintenance pathways were significantly associated with bivalent promoters in HSCs, HPCs, LSCs (and CML LPC too). Bivalent gene promoters carry both activating (H3K4me3) and repressing (H3K27me3) histone marks. Such epigenetic modification is thought to mark genes poised in a bipotential state which can be resolved to a monovalent state that confers a more stable silencing (H3K27me3-only) or transcriptional activation (H3K4me3-only) to their regulated loci, in the course of differentiation and lineage commitment. As such the bivalent epigenetic signature tends to be enriched in developmentally regulated genes[419]. Genes involved in stem cell maintenance pathways play a central role in preserving pluripotency of cells and regulate whether a stem cell undergoes self-renewal or differentiation upon division. Interestingly it was observed in our screen that these genes were specifically enriched for bivalent promoters in both HSC and LSC.

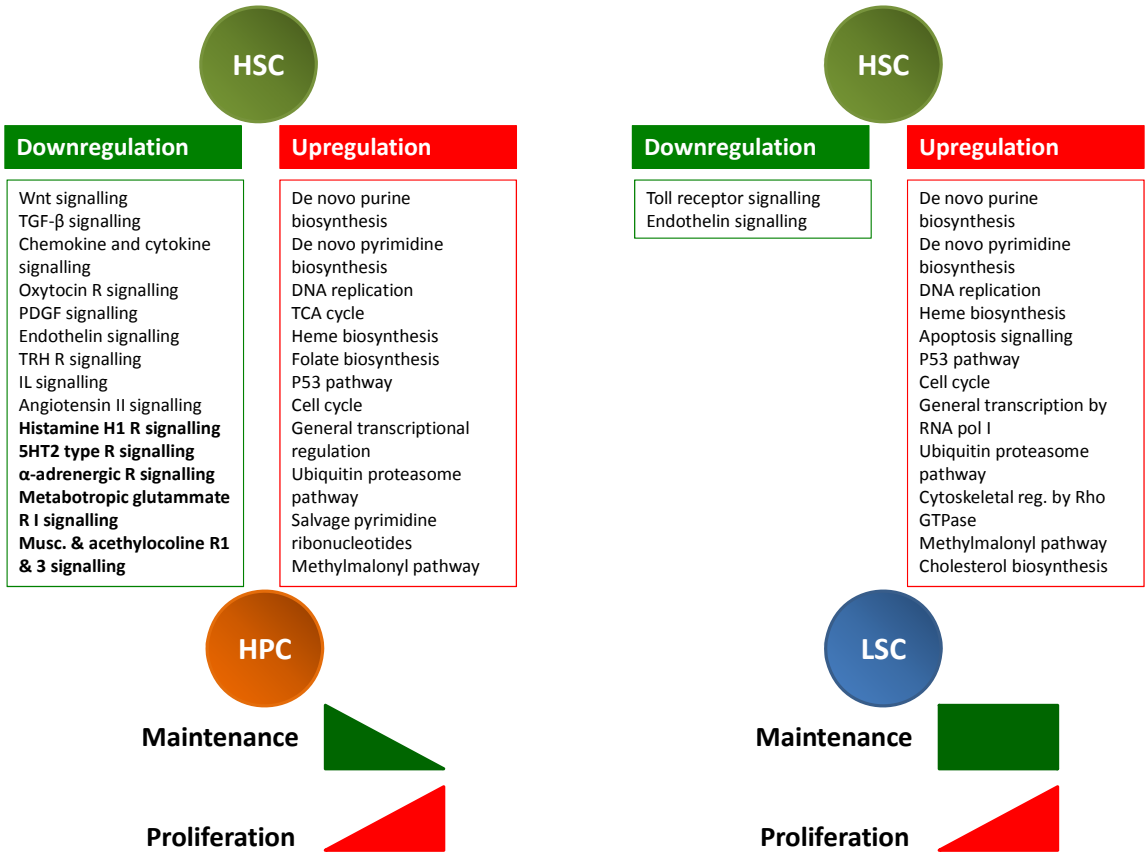


Figure 6-1 Gene expression changes in the transition from HSC to HPC and from HSC to LSC
Pathway analysis of the genes showing significant ($p<0.05$) differential expression in HPCs ($CD34^+$ $CD38^+$) relative to HSCs ($CD34^+$ $CD38^-$) (left) and LSCs ($CD34^+$ $CD38^-$) relative to HSCs (right) (only most biologically relevant pathways from overall analysis listed). A significant upregulation of proliferative pathways in both HPCs and LSCs was observed in comparison to HSCs. Furthermore, several signalling pathways demonstrated significant downregulation in HPCs relative to HSCs which included known stem cell maintenance pathways and some novel pathways possibly involved in stem cell maintenance. Interestingly all these pathways remained unchanged at expression level between HSCs and LSCs comparison. Novel signalling pathways through neurotransmitters potentially involved in the HSC and LSC maintenance are highlighted in bold. The flags at the bottom visually represent the two transitions with maintenance pathways (green) going down from HSC to HPC while proliferative pathways (red) are conversely upregulated. In the HSC to LSC transition although there is an increase in the proliferative pathway expression (red), the maintenance pathways expression (green) does not decrease. This figure was adapted from a figure courtesy of Dr Koorosh Korfi and Dr David Vetrie.

In addition to the putative HSC maintenance pathways several other signalling pathways were also significantly associated with the bivalent promoters in the studied cell types, which included heterotrimeric Gα protein signalling pathways that are core to many neurotransmitter signalling pathways. Consequently, combining the global gene expression and epigenetics analysis several novel neurotransmitter signalling pathways were identified of potential importance in the maintenance of both HSC and LSCs (Dr K. Korfi PhD thesis, University of Glasgow, 2012). These pathways included ACH, catecholamines such as NEPI, 5HT, HIS and GLU signalling pathways. Interestingly investigation of the role of neurotransmitters in normal haemopoietic cell biology, including their self-renewal and

maintenance, has recently been reported. For example catecholaminergic neurotransmitters, such as NEPI, have been shown to increase motility, proliferation and engraftment of human HSPC[108,128], while 5HT has been shown to enhance *ex vivo* expansion of cord blood $CD34^+$ cells, including NOD/SCID mice repopulating cells[129]. Moreover G protein-coupled receptors of neuromediators, such as 5HT, have been shown to be upregulated in human $CD34^+$ $CD38^-$ compared to $CD34^+$ $CD38^+$ cells[126]. A recent report has also shown an indirect role of specific peripheral nervous system cells, namely the non-myelinating Schwann cells, in regulating haemopoietic cell maintenance through regulation of the activation process of TGF-β1[130] (see section 1.1.2.2.2 for more detailed discussion). The role of neurotransmitters in LSC biology is however less clear. Only very recently has it been reported, for example, that inhibitors of dopamine receptors might be able to eradicate LSCs in AML[420], while in CML a single report published several years ago showed upregulation of G protein coupled receptor for neuromediators in CML $CD34^+$ relative to normal $CD34^+$ cells[421], however their functional role was not thoroughly investigated and to date remains unknown.

Therefore the aim of the work presented in the current chapter was to investigate the role of the neurotransmitters whose pathways had been identified by the above described genetic and epigenetic screen in CML LSC biology. In particular their role in CML LSC survival, proliferation and maintenance was assessed using standard *in vitro* cell biology assays such as annexin staining for apoptosis, CFSE staining and CFC replating. Moreover correlative changes in gene expression of genes known to be involved in maintenance and quiescence of LSC was measured by qRT-PCR. Finally the role of TGF-β1 in the same process was also assessed. TGF-β1 was investigated further as, although not differentially expressed between normal HSPC and CML SPC, was amongst the most highly expressed GFs by both CML $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ cells (see 3.1.3) and also appeared to be modulated by some of the neurotransmitters. Considering both the observed autocrine production of TGF-β1 by CML primary SPC described in chapter 3, its modulation by some of the neurotransmitters and the recent reports of its role in CML LSC maintenance in mouse models[111], it was considered interesting to assess if TGF-β1 also played a similar role in the setting of primary human CML LSC maintenance.

6.1 Assessment of the effects of neurotransmitters on CML LSC quiescence and maintenance

6.1.1 Experimental design

When investigating potential regulators of stem cell quiescence/maintenance *in vitro*, it is considered preferable to mimic the BM niche as closely as possible using a stromal coculture system which exposes stem cells to a more physiological environment containing both GFs stimulating their proliferation/differentiation and regulators of quiescence/maintenance. Moreover such a system also allows controlling for any indirect effects of the regulators tested due to the presence of BM stromal cells. This appears particularly important in the case of neurotransmitters as recent evidence suggests that some of the effects of NEPI and 5HT on HSCs and other cell types are secondary to the activation of other known maintenance pathways, such as Wnt[108] or TGF-β1[422]. Although also produced by stem cells, both Wnt and TGF-β1 are secreted by BM stromal cells[100]. Moreover TGF-β1 is normally secreted as a latent protein which requires an enzymatic cleavage and activation by stromal cells[97,130]. Therefore by including BM stromal cells in these experiments, it was conceivable that at least some of these indirect effects would not be missed if present.

OP-9 cells are murine stromal cells originally established from the calvaria of newborn mice genetically deficient in functional macrophage colony-stimulating factor (*M-csf*)[423]. They have been shown to support haemopoietic cell differentiation from both human embryonic stem[424] and induced pluripotent stem cells[425] and have also been routinely used in coculture experiments with human haemopoietic SPC, including CML LSCs[111,426]. Thus there is robust evidence backing their use as a stromal cell line suitable to support haemopoiesis *in vitro*. Therefore to assess the effects of neurotransmitters, CML $CD34^+$ $CD38^-$ cells were cocultured with GFP tagged OP-9 cells for a total of 3 days in the presence or absence of one of the following neurotransmitters: ACH at 100nM, GLU at 10μM, HIS at 100μM, NEPI at 1μM and 5HT at 5μM. All concentrations were chosen based on published work in the literature performed on either haemopoietic cells or other cell systems and showing non toxic effects on the target cells[108,422,427-429]. At the end of the culture period the GFP negative CML LSC were easily identified and separated from the GFP tagged OP-9 stromal cell line by flow cytometry. This allowed both the gene expression and functional assays to be performed, which included CFC and cell

proliferation/apoptosis assays, on a clean population of human CML LSCs (figure 6-2) (more details on the coculture method are available in 2.3.1.5).

Finally it is necessary to mention the reasons behind designing the experiments so that the neurotransmitter pathways were stimulated rather than inhibited in CML LSCs. This does not seem the most logical approach given that CML LSCs have already been shown to be enriched for quiescent and self-renewing cells and therefore detecting an enhancement of quiescence and self-renewal following neurotransmitter treatment might be difficult. However it is worth noting first, that each of the above mentioned neuromediators stimulate different classes of receptors (with both stimulatory and inhibitory effects), hence making it difficult to design an experiment where a pathway can be effectively shut down just by using one inhibitor; secondly, as the source of neurotransmitters in the BM niche are nerve fibres, reproducing an environment containing physiological concentrations of neurotransmitters *in vitro* would be extremely challenging with standard feeder cells which do not include nerve cells. As a result the effects of neurotransmitter receptor inhibitors might be missed in a standard *in vitro* coculture experiment of CML LSC.

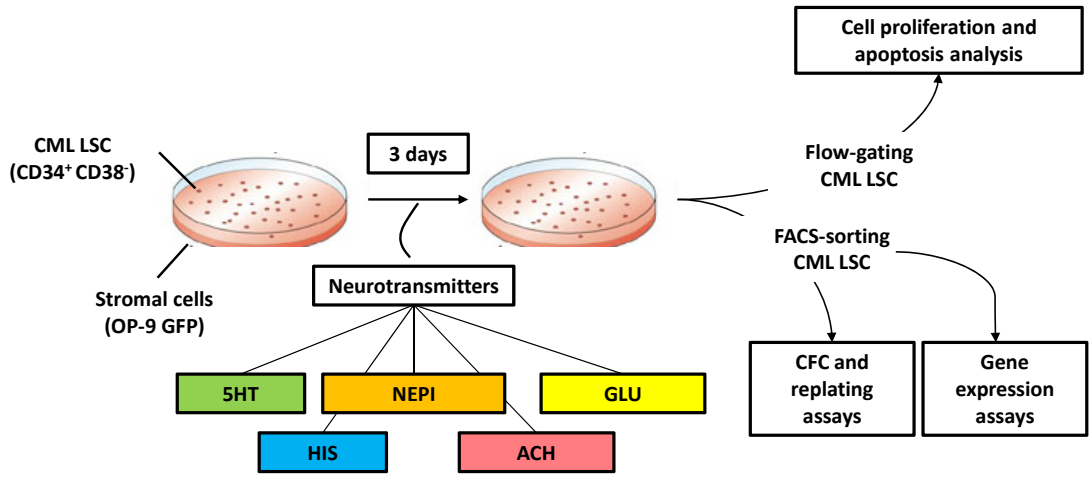


Figure 6-2 Experimental design to assess the effects of neurotransmitters on CML LSC using a coculture system with GFP tagged OP-9

6.1.2 Effects of neurotransmitters on CML LSC proliferation

In order to assess the effects of the neurotransmitters on proliferation, CML LSC were stained with a CellTrace Violet stain after sorting and prior to coculture. This stain has similar properties to the CFSE stain but with different emission spectrum in the violet channel. This made it possible to combine cell proliferation analysis of LSCs cocultured with GFP tagged OP-9 cells which otherwise would have been impossible because GFP has the same emission spectrum as CFSE.

After 3 days in culture in the presence of neurotransmitters at the above mentioned concentrations, there was little difference detected in the overall proliferation of CML LSCs. However more $CD34^+$ cells were recovered in the later divisions of UT samples compared to the treated samples, starting from divisions 3 with more marked differences observed in divisions 4 and 5 (figure 6-3 blue arrows). Conversely the neurotransmitter treated samples had higher recoveries in the earlier divisions (figure 6-3). Overall these data show that in a short term liquid culture the tested neurotransmitters had only mild effects on CML LSC proliferation with a trend towards reduced proliferation seen.

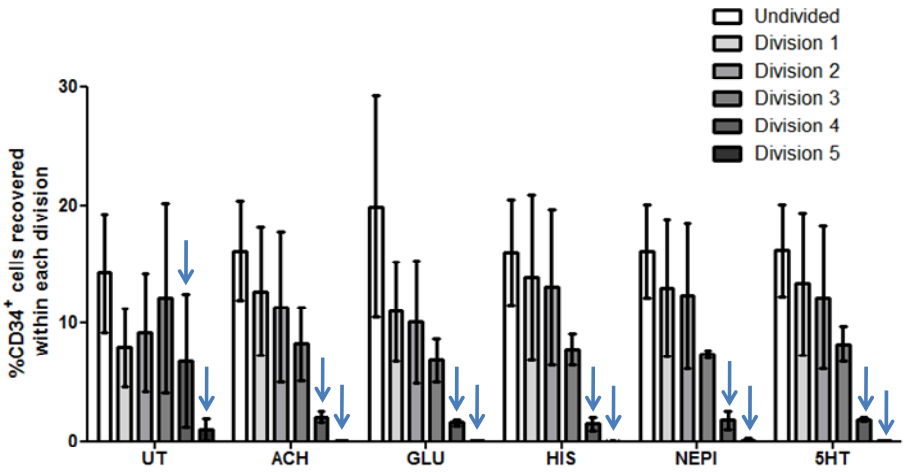


Figure 6-3 Effects of neurotransmitters on CML LSC proliferation
CML LSC ($CD34^+$ $CD38^-$) (n=3) were stained with CellTrace Violet and then cultured for 3 days on an OP-9 stromal layer with ACH (100nM), GLU (10µM), HIS (100µM), NEPI (1µM) and 5HT (5µM) or left UT before flow cytometry analysis. Percentages of starting $CD34^+$ cells recovered within each division following treatment were calculated as explained in 2.3.2.3.

6.1.3 Effects of neurotransmitters on CML LSC viability and apoptosis

Next the effects of neurotransmitters on CML LSC viability and apoptosis were assessed. To this end the total number of $CD34^+$ cells recovered in the UT and treated samples at the end of the 3 day coculture period was measured. Apoptosis levels were also recorded using standard annexin-V staining.

Neurotransmitters did not affect CML LSC viability or apoptosis in short term cultures as both total $CD34^+$ cells recovered (figure 6-4 A) and apoptosis levels (6-4 B) following treatment were not different compared to UT samples. Overall these data show that neurotransmitters, at least at the concentrations used in these experiments, are not toxic to CML LSCs.

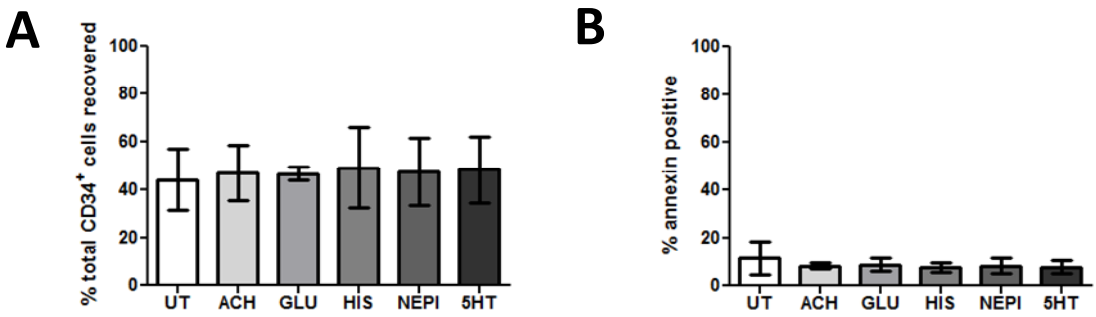


Figure 6-4 Effects of neurotransmitters on CML LSC viability and apoptosis
CML LSC ($CD34^+$ $CD38^-$) (n=3) were stained with CellTrace Violet stain and then cultured for 3 days on an OP-9 stromal layer with ACH (100nM), GLU (10µM), HIS (100µM), NEPI (1µM) and 5HT (5µM) or left UT before flow cytometry analysis. Percentages of the starting $CD34^+$ cells recovered in total following treatment were calculated as explained in 2.3.2.3 (A). At day 3 CML LSC were also stained with annexin-V and total apoptosis measured within each treatment arm (B).

6.1.4 Effects of neurotransmitters on CML LSC CFC output and replating

In order to assess the effects of neurotransmitters on CML LSC self-renewal capacity, CFC replating assays were performed following the 3 day coculture experiments by plating between 1,000 to 3,000 (depending on cells recovered at the end of the experiment) CML LSCs in methylcellulose assays. After 10 to 12 days CFC were enumerated for each arm and thereafter single colonies harvested and resuspended in fresh methylcellulose for further 12 to 14 days prior to colony counting. The first round of such CFC assay (CFC 1) is considered a readout for the haemopoietic progenitors' ability to proliferate and differentiate. However by assessing the morphology of the colonies produced (erythroid such as CFU-E and BFU-E versus myeloid and mixed such as CFU-GM) it is possible to infer the type of progenitors present in the sample, with erythroid colonies normally considered a reflection of a more mature progenitor cell compared to myeloid colonies[343]. Interestingly amongst the neurotransmitters, ACH and NEPI appeared to increase the number of CFC 1 produced (figure 6-5 A), mainly as a result of increased presence of myeloid and mixed colonies (figure 6-5 B). However the differences did not reach statistical significance. When the capacity of these colonies to replate in secondary CFC assays (CFC 2) was measured, it became clear that both NEPI and ACH increased the replating efficiency of CML LSC compared to UT ($p<0.05$ for NEPI versus UT) with a minor trend towards a similar effect for 5HT (figure 6-5 C). Morphologically, this increase was due mainly to a higher number of erythroid colonies in the treated samples ($p<0.05$ for both ACH versus UT and NEPI versus UT), which was expected as by the second round of CFC, cells had been cultured for over 4 weeks and possibly undergone a degree of differentiation. However an increased number of myeloid colonies was also produced in CFC 2 following NEPI treatment (and to a lesser extent following ACH treatment) (figure 6-5 D). Finally the size of the colonies produced by both NEPI and ACH in CFC 2 was bigger compared to UT (figure 6-6 E).

Given that the capacity of an individual haemopoietic progenitor cell colony to replate in a CFC assay has been used as an indicator of the self-renewal potential of that progenitor[344,345], these data show that NEPI, ACH and possibly 5HT (although to a lesser extent) increase the self-renewal potential of CML LSCs and thus contribute to their maintenance.

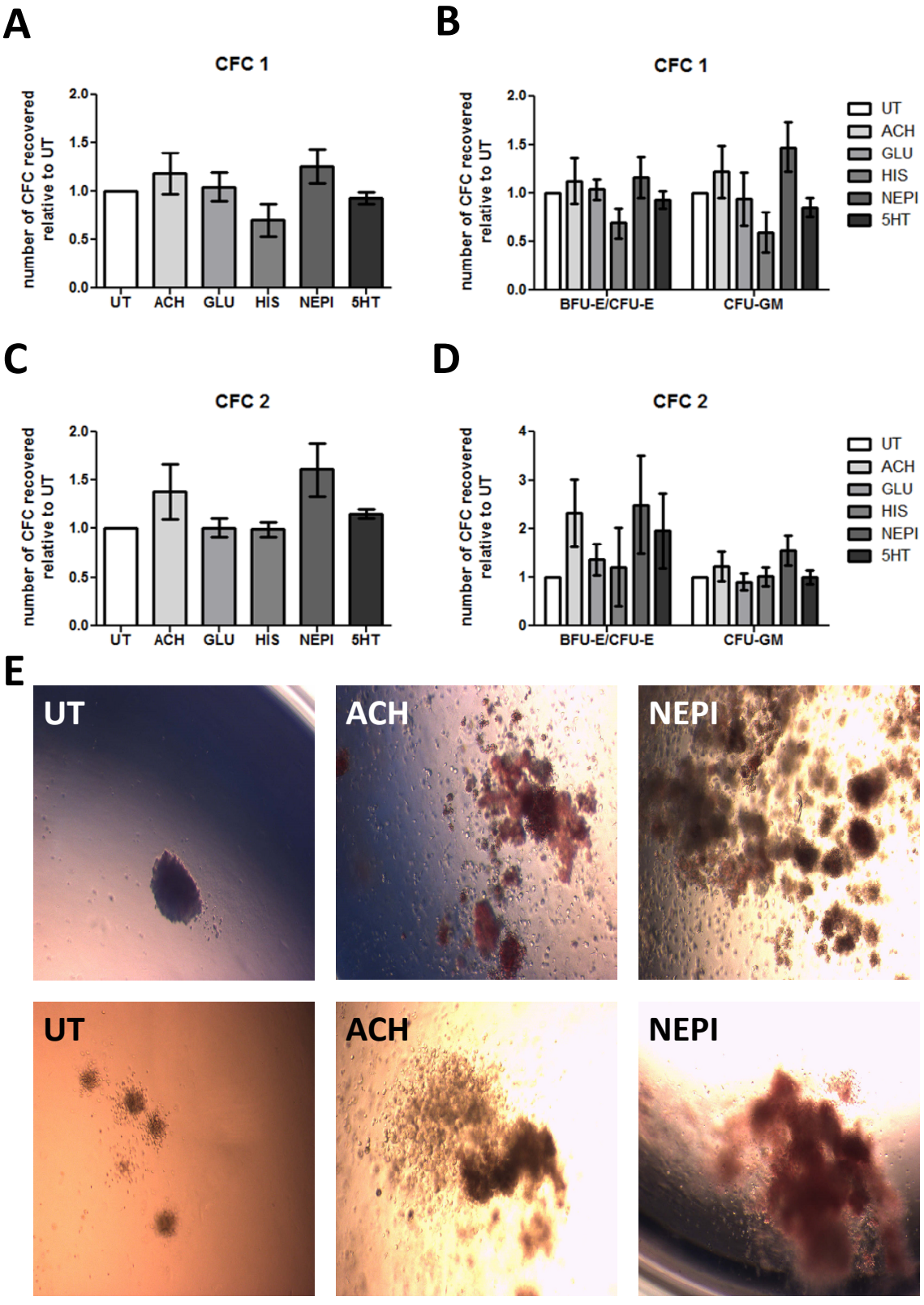


Figure 6-5 Effects of neurotransmitters on CML LSC CFC output and replating
CML LSC ($CD34^+$ $CD38^-$) (n=3) were cultured on an OP-9 stromal layer for 3 days with ACH (100nM), GLU (10μM), HIS (100μM), NEPI (1μM) and 5HT (5μM) or left UT before between 1,000 to 3,000 cells from each of the arms were plated in standard CFC. Number of colonies recovered in total and based on their morphology in CFC 1 (A and B) and CFC 2 (C and D) are plotted. Representative images of replated CFC from UT, ACH and NEPI treated cells (E). As the starting number of cells plated in CFC was different in each of the 3 samples, the number of CFC obtained was expressed relative to UT in each sample. For CFC1 the number of colonies recovered in each arm was adjusted by the CML LSCs expansion after 3 days *in vitro* culture.

6.1.5 Investigation into the activity of neurotransmitter signalling pathways in CML LSC

As already mentioned in the chapter introduction, the heterotrimeric Gα protein signalling pathways, which are core to many neurotransmitter signalling pathways and in particular to NEPI and ACH signalling, were shown to be significantly associated with the bivalent promoters in the epigenetic screen of HSC and LSC. Therefore it was felt necessary to investigate if any of these signalling pathways were active and modulated by the neurotransmitters tested, in particular NEPI and ACH being the ones which had produced the most significant biological effects and known to mainly signal through the Gα protein signalling pathways. Based on the original genome-wide and epigenetic screen carried out in our laboratory, the pathways which appeared to be activated in LSC maintenance were mainly the α adrenergic receptor signalling pathway for NEPI and the muscarinic receptor 1 and 3 signalling pathway for ACH (figure 6-1). Both α adrenergic receptor and muscarinic 1 and 3 receptors are part of the G protein coupled receptor family and their signalling pathways have been reviewed in the Introduction section 1.1.2.2.2. Several components of these pathways were analysed. Amongst the receptors both the muscarinic receptor 3 (*CHRM3*) and the α2 adrenergic receptor (*ADRA2A*) were detected in CML LSC with *ADRA2A* levels also being upregulated by exposure to ACH and NEPI. These receptors activate the G_i and G_q subtypes of Gα proteins and, through G_q, PLCB1. PLCB1 activates several isoforms of PRKC. Amongst those, PRKC epsilon (PRKCE) has emerged as a possible oncogene in several solid tumours[430,431]. Interestingly *G_i*, *PLCB1* and *PRKCE* gene expression levels appeared upregulated following NEPI and ACH stimulation in CML LSCs (figure 6-6). Taken together these results suggest that some of the signalling pathways modulated by both NEPI and ACH are present and active in CML LSCs and confirm that neurotransmitters can also generate signals in haemopoietic stem cells.

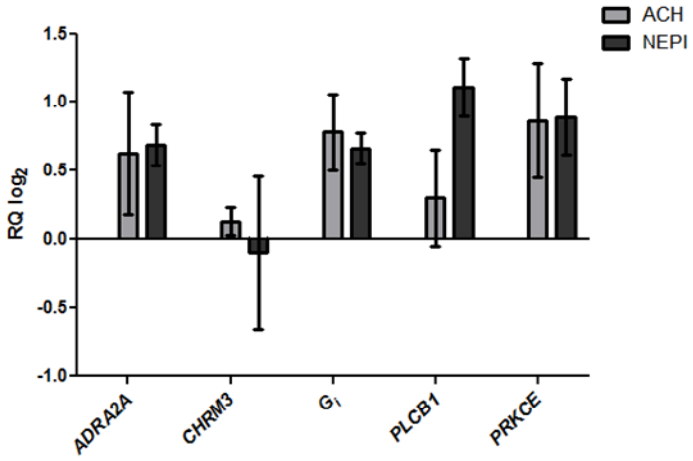


Figure 6-6 Gene expression changes of components of neurotransmitter signalling pathways in CML LSC following exposure to NEPI and ACH
Gene expression changes for target genes were measured in CML LSC (n=3) samples following 3 days coculture on an OP-9 stromal layer in the presence of ACH (100nM), NEPI (1μM) or no drug. Differences in gene expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator).

6.1.6 Effects of neurotransmitters on gene expression levels of regulators of quiescence and maintenance

After showing that NEPI and ACH can activate specific neurotransmitters signalling pathways, gene expression changes in key regulators of quiescence and maintenance of CML LSCs were measured following all 5 neurotransmitter treatments. Interestingly consistent correlative changes in genes involved in haemopoietic stem cell quiescence and maintenance of CSC and LSC in general, such as *cyclin D1*, *CDKN1A/p21* and *ID1* were only seen following NEPI treatment and to a lesser extent ACH and 5HT treatment (figure 6-7 A). Moreover upregulation of *TGF-β1* and its cognate receptors *TGF-β1 R1* and *R2* gene expression was also seen only following NEPI and ACH treatment (figure 6-7 B). TGF-β1 has recently been shown mainly in mouse models to play a key role in CML LSC maintenance[111]. It is worth noting that similar changes in gene expression levels were not observed following HIS and GLU treatment and only to a reduced level following 5HT treatment. This supports the conclusion that the correlative gene expression changes observed in these experiments were important to the biological effects seen following NEPI and ACH treatment as they were not observed following treatment with the neurotransmitters which had not elicited significant biological responses.

Overall these data support the conclusion that NEPI and ACH activate known pathways central to LSC and HSC quiescence/maintenance. Whether these effects are directly caused by the activation of their signalling pathways or through upregulation of other mediators cannot be inferred based on these data. However the upregulation of *TGF-β1* and its receptors is suggestive of a role for this cytokine in the effects seen following NEPI and ACH treatment.

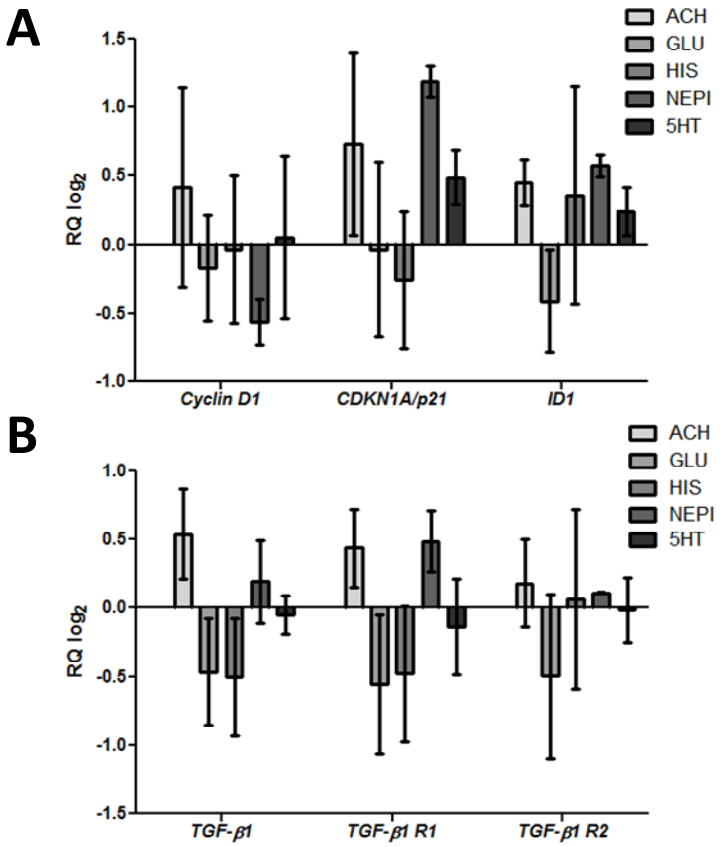


Figure 6-7 Gene expression changes in regulators of LSC quiescence/maintenance following neurotransmitter treatment

Gene expression changes for genes central to HSC and LSC quiescence and maintenance (A) and *TGF-β1* and its receptors (B) were measured in CML LSC samples (n=3) following 3 days coculture on an OP-9 stromal layer in the presence of ACH (100nM), GLU (10μM), HIS (100μM), NEPI (1μM), 5HT (5μM) or no drug. Differences in gene expression levels following treatment were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of two housekeeping genes (*GAPDH* and *TBP*). RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator).

6.2 Assessment of the effects of TGF-β1 on CML LSC quiescence and maintenance

TGF-β1 is a cytokine with pleoiotropic effects which vary depending on the cell context on which it acts[109]. It has been shown to act as a negative regulator of HSC proliferation *in vitro* although its effects can be different on more mature progenitors[112-114] (see section 1.1.2.2.1 for detailed discussion). In CML stem cell biology, the role of TGF-β1 has been investigated for a long time. Most of the older reports showed, using long term culture of primary CML MNC, that CML SPC are still responsive to the inhibitory effects of TGF-β1 on proliferation. However they did not investigate the role of TGF-β1 in CML LSC maintenance, but focused on trying to understand why primary CML SPC displayed a highly proliferative phenotype despite being responsive to TGF-β1[259,432]. More recently a report using BCR-ABL positive cell lines and primary CML $CD34^+$ cells from CP patients showed higher sensitivity of BCR-ABL positive cells to TGF-β1 signalling and that inhibition of TGF-β1 signalling, together with IM, increased the kill of CML $CD34^+$ cells, possibly by inducing quiescent cells into cycle, although this was not definitively proven[433]. Consistent with this, Naka *et al* have very recently demonstrated a central role for TGF-β1 in CML LSC maintenance through its inhibition of AKT signalling and concomitant activation of FOXO3a transcription factors in a CML mouse model. They also show that inhibition of TGF-β1 signalling together with IM led to prolonged survival of mice affected by CML[111], which also suggests that this pathway is more relevant to CML LSC maintenance than normal HSC maintenance. Therefore it appears that inhibiting the TGF-β1/AKT axis might lead to preferential CML LSC exhaustion. However very little work on the role of TGF-β1 in survival, proliferation and maintenance of an enriched population of primary human CML LSCs ($CD34^+$ $CD38^-$) has been performed, while nothing is known of the pathways regulated by TGF-β1 in the same population. Working on a pure or at least enriched population is essential when trying to understand TGF-β1's role in CML LSC biology as TGF-β1's effects on CML cells are very dependent on the cell context, as shown by another report which demonstrates that silencing TGF-β1 signalling by BCR-ABL oncoprotein is necessary to the survival of CML BC derived cell lines[250]. In the next section the effects of TGF-β1 in controlling human CML LSCs cell cycle regulation, survival and signalling was investigated and compared to its effects on normal HSCs. Finally using the more physiological OP-9 coculture *in vitro* model, a strategy targeting TGF-β1 signalling in primary CML LSCs was assessed for its effects on proliferation, maintenance and signalling.

6.2.1 Effects of TGF-β1 on CML LSC and normal HSC cell cycle, apoptosis and signalling

In a first set of experiments the effects of TGF-β1 in inducing quiescence and apoptosis and on signalling in CML and normal $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ populations were assessed. Although both CML LSCs and normal HSCs produce autocrine TGF-β1, this might not be active in a liquid *in vitro* culture system in the absence of stroma[97,111]. This is because TGF-β1 is secreted as a latent protein which requires activation by the stroma[110]. Therefore in these experiments TGF-β1 was supplied exogenously to CML and normal $CD34^+$ cells cultured *in vitro* in SFM+PGF at a concentration of 5ng/mL, a concentration chosen based on published studies in the literature[433], but above all shown to be present in the BM stroma of CML patients[275]. AKT phosphorylation levels on threonine 308 (a known activation marker of this protein) were then measured at 8 hours following start of culture by intracellular flow cytometry, while high resolution cell cycle analysis (see 2.3.2.2) and standard annexin-V staining were performed at 24 hours. All these assays were combined with surface staining to allow characterisation of the effects within phenotypically defined subpopulations of SPC.

TGF-β1 increased the number of cells in G0 in both CML populations to a similar extent (by about 10%) (figure 6-8 A). Although a higher proportion of normal HSCs were quiescent compared to CML LSCs (93% versus 52% respectively) no further induction of quiescence was seen in normal HSCs and only a mild increase (about 6%) of the G0 population was seen in normal HPCs at 24 hours (figure 6-8 B). While a variable degree of apoptosis induction was detected in CML LPCs and normal HSCs and HPCs, no significant induction of apoptosis was seen in CML LSCs following TGF-β1 treatment at 24 hours (figure 6-8 C and D). Although CML LSCs expressed lower levels of p-AKT compared to CML LPCs, its levels were further reduced by TGF-β1 (figure 6-8 E). Conversely both normal HPCs and HSCs expressed barely detectable levels of p-AKT (just above isotype control levels) which were only mildly affected by TGF-β1 (figure 6-8 F).

These data confirm that TGF-β1 induces quiescence of CML LSCs within 24 hours without causing concurrent apoptosis. This effect is at least partially secondary to downregulation of AKT signalling based on the correlative changes in p-AKT levels following TGF-β1 exposure. Conversely normal HSCs express very low levels of p-AKT, are mostly quiescent and not induced into further G0 arrest by TGF-β1. Taken together these results suggest that CML LSCs are more sensitive than normal HSC to TGF-β1

induced quiescence and could therefore be more dependent on TGF-β1 signalling for their maintenance.

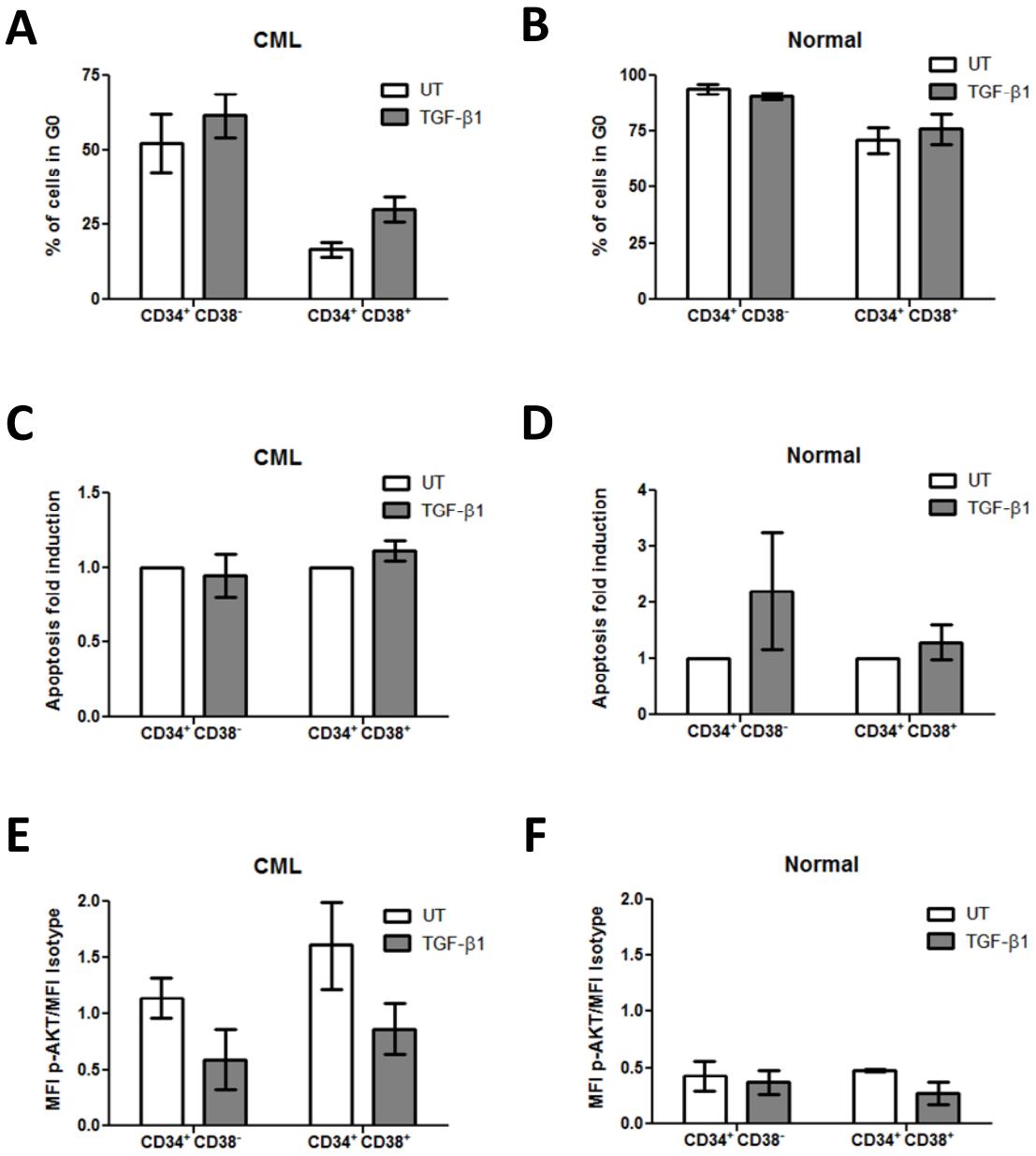


Figure 6-8 Effects of TGF-β1 on CML SPC and normal HSPC cell cycle, apoptosis and signalling

CML CD34$^+$ (n=3) and normal CD34$^+$ (n=2) cells were cultured in SFM+PGF in the presence of TGF-β1 at 5ng/mL or no drug. At 24 hours cells were then first stained for CD34 and CD38 and thereafter high resolution cell cycle analysis was performed as explained in 2.3.2.2 in CML (A) and normal (B) samples. Apoptosis was measured by annexin-V staining in CML (C) and normal (D) samples. P-AKT levels were measured at 8 hours by intracellular flow cytometry in the same CML (E) and normal (F) samples. Levels of p-AKT were calculated based on MFI of antibody stained samples relative to isotype stained samples.

6.2.2 Effects of inhibition of TGF-β1 signalling on CML LSC quiescence, self-renewal and signalling

In order to investigate the effects of inhibition of TGF-β1 signalling on CML LSC proliferation, self-renewal and signalling, experiments were undertaken using the OP-9 stromal coculture method previously described (see 6.1.1). The presence of stroma in this system would allow for CML LSC autocrine produced TGF-β1 to be activated so that the effects of modulating the TGF-β1 signals would be assessed in a more physiological situation. Overall as previously mentioned, this also represents a better model to study mechanisms regulating quiescence *in vitro* as CML LSCs would be exposed to both positive and negative regulators of haemopoiesis. It should be noted that in the previous liquid culture experiments carried out in the presence of PGF (i.e. mainly positive regulators of haemopoiesis) almost 50% of CML LSCs were in cycle after 24 hours which confirms the inadequacy of such a system to study regulators of quiescence and the effects of their pharmacological modulation.

CML $CD34^+$ $CD38^-$ cells were therefore sorted and cultured on an OP-9 stromal layer with no drug or in the presence of the TGF-β1 R1 kinase inhibitor LY at 10μM as previously published[111]. Effects on proliferation and recovery of cells were then assessed using the CellTrace Violet stain as previously described (6.1.2). After 3 days in culture in the presence of LY there was a reduction in the recovery of undivided CML LSCs together with an overall reduction in $CD34^+$ cells recovered (figure 6-9 A). These data show that inhibition of TGF-β1 signalling causes a reduction in the quiescence of CML LSCs with concurrent reduction in their overall survival. Consistent with this LY decreased the number of CFC 1 produced compared to UT with similar effects on both erythroid and myeloid/mixed colonies. These effects were confirmed also in CFC 2 as LY reduced the replating efficiency of CML LSCs compared to UT (figure 6-9 B). These data show that inhibition of TGF-β1 signalling reduces the self-renewal potential of CML LSCs. Finally, the effects seen following LY treatment correlated with downregulation of the gene expression of *CDKN1A/p21*, *CDKN1B/p27* and *PTEN*, all key regulators of HSC quiescence/maintenance normally activated by TGF-β1, and upregulation of the growth promoting transcription factor *MYC* normally inhibited by TGF-β1[117](figure 6-9 C). Overall these data suggest that inhibition of the TGF-β1 pathway reduces quiescence and self-renewal of CML LSCs, possibly through downregulation of *CDKN1A/p21*, *CDKN1B/p27* and *PTEN* and upregulation of *MYC* and confirm in primary human CML samples the role of the TGF-β1 signalling in the maintenance of CML LSCs.

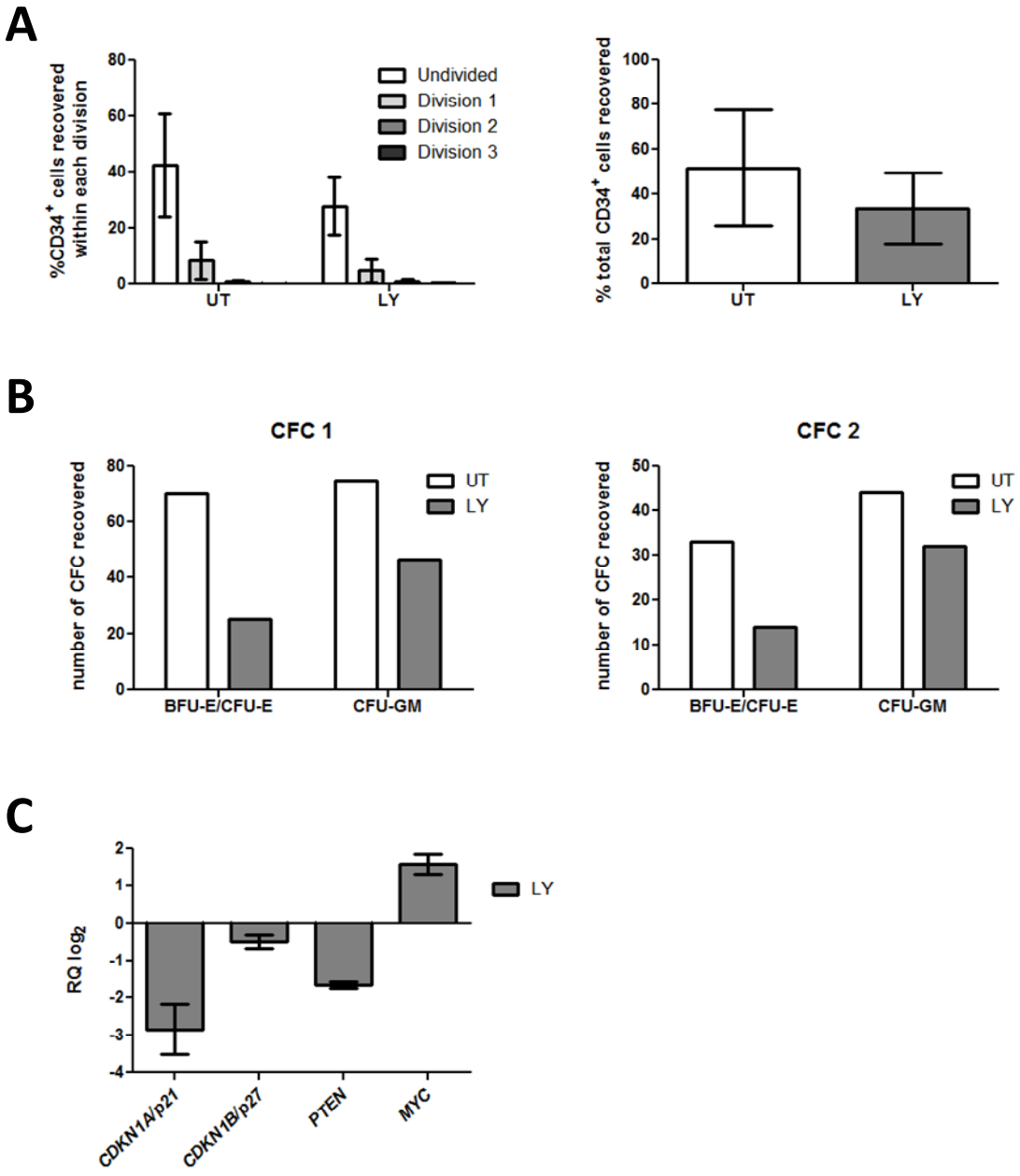


Figure 6-9 Effects of inhibition of TGF-β1 signalling on CML LSC quiescence, self-renewal and signalling

CML LSC ($CD34^+$ $CD38^-$) (n=2) were stained with CellTrace Violet stain and then cultured for 3 days on an OP-9 stromal layer with LY (10μM) or left UT before flow cytometry analysis. Percentages of the starting $CD34^+$ cells recovered within each division and in total following treatment were calculated as explained in 2.3.2.3 (A). After 3 days in culture in the same conditions as before, 2,000 cells from each of the arms were plated in standard CFC (n=1). Number of colonies recovered in total and based on their morphology in CFC 1 and CFC 2 are plotted (B). For CFC1 the number of colonies recovered in each arm was adjusted by the CML LSCs expansion after 3 days *in vitro* culture. Gene expression changes for *CDKN1A/p21*, *CDKN1B/p27*, *PTEN* and *MYC* in LSC samples (n=2) following 3 days coculture on an OP-9 stromal layer in the same conditions as above were calculated using the $2^{-\Delta\Delta Ct}$ method after normalisation within each sample of candidate gene expression levels against the expression levels of the housekeeping gene *GAPDH*. RQ of candidate gene mRNA expression following each treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator).

6.3 Summary and future directions

6.3.1 Novel putative role of NEPI and ACH in CML LSC maintenance

The work presented in this chapter aimed to investigate novel autocrine and paracrine cues involved in CML LSC maintenance. It originated from an unbiased genetic and epigenetic screen which suggested a potential role for several neuromediators signalling pathways in maintenance of CML LSC. In order to validate these findings a set of *in vitro* experiments of primary CML LSCs ($CD34^+$ $CD38^-$) were designed using a stromal coculture setup with OP-9 cells, to mimic more closely the BM niche. Studying stem cell function in *in vitro* assays is inevitably limited and needs to be complemented with *in vivo* experiments, such as repopulating assays of immunodeficient host, which is an obvious limitation of the investigations presented. Nevertheless interesting findings were generated as a result of these *in vitro* experiments.

None of the neuromediators tested, at the concentrations used in the experiments, were toxic to CML LSCs. Their effects on CML LSC proliferative ability in short term liquid culture experiments were mild as only a slight reduction of the expansion in cells in later divisions relative to UT cells was observed with no obvious effects on the undivided population. However, an effect on CML LSC self-renewal based on replating capacity was observed for two of the neuromediators tested, i.e. NEPI and ACH. This was also associated with a mild increase in colony forming ability after short term culture in the presence of NEPI and ACH with a relative increase in more primitive colonies, such as CFU-GM. It is worth noting that due to limited cell numbers and the necessity to screen multiple neurotransmitters, it was not possible to titrate the concentration at which all the tested neuromediators were used so the concentrations used in the experiments were based on published work often in different cell model systems[108,422,427-429]. It is therefore possible that in some cases either inadequate or excessive concentrations were used which might explain the lack of effects seen apart from NEPI and ACH. In this respect it is interesting however to note that NEPI was the only neuromediator for which a study on its effects on primary HSCs had been published and therefore its concentration was chosen based on effects seen on a similar model system[108]. This might be one reason why NEPI was the neuromediator yielding more positive results. Also amongst all the neuromediators tested, NEPI is the one which has been linked more to HSC function (see section 1.1.2.2.2) so far, so it is possible that it is the one with most relevant biological function in this

setting[108,127,128]. Moreover the heterotrimeric Gα protein signalling pathways, which were shown in the epigenetic screen to be significantly associated with the bivalent promoters in the CML LSC, are the most important signalling pathways downstream of NEPI and ACH, although they also play a role to a lesser extent in the signalling of the other neuromediators tested[123]. All these considerations might help to explain why amongst all neuromediators tested, NEPI, and to a lesser extent ACH, appeared to produce the most biologically relevant results.

The possible mechanisms of the effects seen were studied by analysing gene expression changes following 3 days culture of CML LSCs in the presence of neurotransmitters. The heterotrimeric Gα protein signalling pathways, activated by both NEPI and ACH, were shown to be present and active in CML LSCs upon stimulation with these neuromediators. This finding reinforces the hypothesis that heterotrimeric Gα protein signalling pathways activated via neuromediators present in the BM niche might indeed play a role in CML LSC maintenance. Moreover upon stimulation with neuromediators other genes more commonly involved in HSC and also LSC quiescence/maintenance, such as *CDKN1A/p21*[95] and *ID1*[380], were shown to be upregulated, which provides a potential mechanism for the effects seen. Reassuringly the effects on these genes were only observed following treatment with NEPI and ACH which, as mentioned, were the only two neuromediators clearly showing an effect on CML LSC self-renewal. This result therefore further validates the effects seen following NEPI and ACH treatment and also suggests that the correlative changes in these genes expression might be relevant to the biological effects seen. Moreover the upregulation of both *TGF-β1* (mainly for ACH) and *TGF-β1 R1* (for both NEPI and ACH) observed would suggest that some of the effects seen might be secondary to upregulation of the TGF-β1 pathway in CML LSC.

The potential role of NEPI and ACH in CML LSC maintenance is a completely novel finding and shows the value of using unbiased screens to highlight otherwise unknown mechanisms, in this case, potentially relevant to the maintenance of CML LSC. How does this fit with what is already known in the literature about neuromediators and haemopoiesis? As discussed in the Introduction (section 1.1.2.2.2), only recently a role for neuromediators, and in particular NEPI, in different aspects of HSC biology has been shown. This included a role for NEPI in indirectly regulating HSC egress from the BM by suppressing endosteal osteoblast function and leading to reduction in the levels of a key chemokine involved in HSC BM retention, SDF1[127]. Also treatment of human $CD34^+$ cells with NEPI (similar to what was done in CML LSC in the work presented, except that

here an even more pure stem cell subset i.e. $CD34^+$ $CD38^-$ was tested) has been shown to increase their mobilisation, proliferation and repopulation capacity in immunodeficient hosts[108,128]. The effects of NEPI on CML LSC migration were not tested and although the effects seen on short term liquid culture were consistent with a mild antiproliferative effect, in the first round of CFC both NEPI and ACH already produced an increased colony output relative to UT, which suggests that indeed these neurotransmitters increased proliferative potential of CML LSC. The fact that this effect was carried over into CFC replating suggests that these proliferative effects were also associated with maintenance of self-renewal capacity and again would be consistent with the increased repopulating capacity seen in normal HSCs exposed to the same agent. In summary, it appears that NEPI, and ACH to a lesser extent, increase the proliferative capacity of primitive CML LSCs, while maintaining their self-renewal capacity.

Although the effects of NEPI and other neuromediators in CML cells have never been published before, it is worth mentioning that a single report published several years ago showed upregulation of G protein coupled receptors for neuromediators in CML $CD34^+$ relative to normal $CD34^+$ cells[421], although their functional role was not investigated. More recently in AML a putative role for dopamine activated pathways has been shown in maintenance of LSC. A dopamine receptor antagonist was shown to reduce LSC engraftment in irradiated hosts and to reduce viability and CFC output of both AML cell lines and primary AML samples, while the opposite effects were seen when the dopamine receptor was stimulated. Moreover this could be combined with standard cytotoxic treatment (cytarabine) leading to almost complete elimination of CFC output at low, non toxic concentrations of cytarabine[420]. Although dopamine signals via different receptors compared to NEPI and ACH, it is a precursor of NEPI and overall these findings are supportive of the putative role of neuromediator signalling pathways in haematological malignancies and LSC survival (figure 6-10).

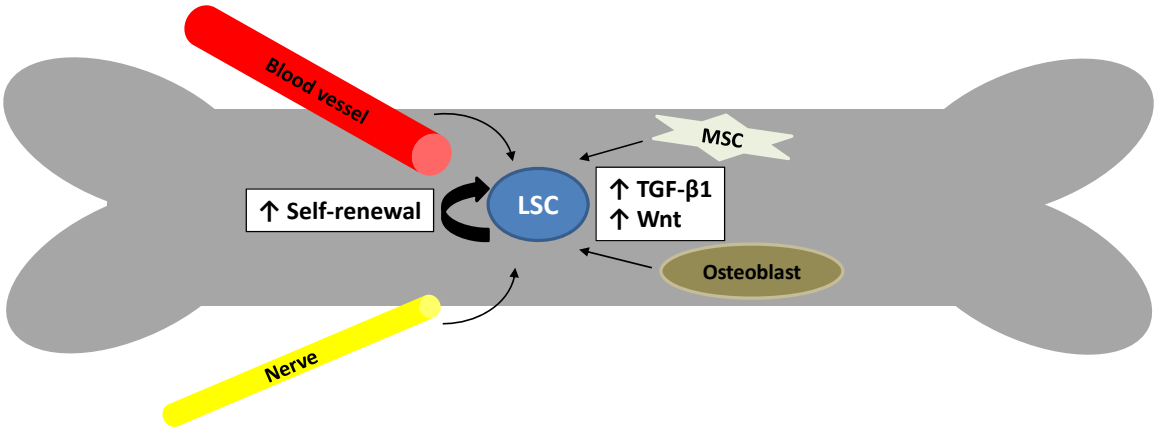


Figure 6-10 Model for control of LSC self-renewal by different cell types in the BM niche
LSC are exposed to several different cues from different cell types in the BM niche to control their self-renewal. A role for the nervous system has only recently being elucidated and appears to act via direct and indirect effects such as possibly stimulation of other pathways including Wnt and possibly TGF-β1. Abbreviations: MSC, mesenchymal cell.

In conclusion, although preliminary, these findings are suggestive of a potential role of neuromediators, in particular NEPI, in CML LSC maintenance, which could be targeted for therapeutic purposes. However the limitations of the work presented should also be taken into account. Although $CD34^+$ $CD38^-$ cells represent an enriched population of CML LSCs, they still contain a significant number of progenitors and therefore it is necessary to take this into account when discussing the role of neurotransmitters on CML LSC maintenance based on the experiments done. In fact, as already mentioned, the best way to confirm an effect on CML LSC is by functional *in vivo* assays. It might well be that using more stringent functional assays to assess maintenance of CML LSC, some of the effects seen could be amplified if these are truly CML LSC specific effects. Also, as already mentioned the mechanisms of action for NEPI and ACH are still rather speculative and require further experimental evidence. In particular the involvement of TGF-β1 in the effects seen should be investigated by assessing the effects of NEPI on p-AKT and p-SMAD levels and also FOXO activity in more detail. It might also be worth testing if the inhibition of the TGF-β1 pathway would reverse the effects seen with NEPI and ACH, including any effects detected at the molecular level. Moreover it is worth noting that in normal HSC a role for Wnt/β-catenin in the effects seen following NEPI stimulation[108] had been proposed and this avenue might also be worth investigating. Eventually the role of these neuromediator related pathways in normal HSC maintenance versus CML LSC maintenance should be compared directly as this might clarify if these pathways could be exploited therapeutically. As is often the case in CML, there is a great degree of overlap between the pathways relevant to CML LSC and normal HSC maintenance and therefore

unless CML LSCs are proven to be more dependent on them, their therapeutic exploitation might be limited.

6.3.2 TGF-β1 induces quiescence in primary CML LSC via downregulation of p-AKT levels and its effects can be reversed through a specific inhibitor

The investigations on TGF-β1 in primary CML LSC were prompted by a variety of reasons. TGF-β1 has long been known to act as a negative regulator of both normal HSC and LSC proliferation. Moreover these effects on proliferation have only recently been linked to an increase in repopulating capacity of both normal HSC and CML LSC in mouse models. The observed upregulation of both TGF-β1 and its receptors following NEPI and ACH treatment of CML LSCs also sparked further interest in this pathway as a major regulator of primary CML LSC maintenance. While the effects in both normal and CML mouse models of TGF-β1 have recently been reported, its effects in the complementary model of primary CML LSCs has not been as thoroughly investigated. Moreover while in mouse models its mechanism of action has been linked to inactivation of AKT and consequent upregulation of FOXO3a activity[111], the mechanisms underlying its putative effects in human primary CML LSC are still unknown. Finally a direct comparison of the sensitivity to TGF-β1 effects in normal HSC and CML LSC was felt necessary to clarify if these two cell types respond differently and therefore if this pathway could be therapeutically exploited. To clarify these issues different sets of experiments were performed, including *in vitro* culture experiments in the presence of exogenous TGF-β1 and stromal coculture experiments with OP-9 cells to more closely mimic the BM niche.

The results of the experiments performed showed that TGF-β1 is capable of inducing G0 arrest in CML LSCs to a higher degree than in normal HSCs. These effects were not associated with toxic effects to the same cells and correlated with downregulation of AKT activity. In the stromal coculture experiments, inhibition of TGF-β1 signalling was associated with a reduction in undivided cells recovered relative to UT and also a reduction of overall cells recovered. These results suggested a role for TGF-β1 in preserving CML LSC quiescence and also their overall viability. Upon TGF-β1 R1 kinase inhibition, both CML LSC clonogenic potential and self-renewal capacity were reduced. All these effects correlated with biologically consistent gene expression changes as negative cell cycle regulators, normally induced by TGF-β1, such as *CDKN1A/p21* and *CDKN1B/p27*, were downregulated by the TGF-β1 R1 kinase inhibitor, while the opposite effects were

observed for a positive regulator of cell cycle, such as *MYC*, normally downregulated by TGF-β1 signalling[117].

All the above findings are consistent with those recently reported by Naka *et al* who studied the role of TGF-β1 in CML LSC maintenance using mouse models[111] and support their conclusion in the complementary primary human CML LSC model system. Moreover they support the hypothesis that upregulation of TGF-β1 signalling might contribute to the biological effects seen following NEPI and ACH stimulation of CML LSCs. The overall downregulation of AKT signalling, as shown by both reduced AKT phosphorylation following TGF-β1 treatment and downregulation of *PTEN* gene expression levels upon treatment with the TGF-β1 R1 kinase inhibitor LY, support the hypothesis that AKT signalling plays a central role in the mechanism of action of TGF-β1 and its effects on CML LSC. The ability of TGF-β1 to reduce AKT signalling in HSC, possibly through its effects on lipid raft clustering[97], has been already reported and discussed in the Introduction (see section 1.1.2.2.1). It is interesting to note that recently several reports have shown that phenotypically defined murine and human CML LSCs have lower levels of p-AKT compared to CML LPCs[111,338], despite the fact that mRNA and protein expression of BCR-ABL oncoprotein appears to be even higher in the LSC subsets as previously reported[329,434] (also personal data not shown). The findings from the experiments presented are therefore consistent with those already reported and with a model whereby although BCR-ABL protein and mRNA expression is increased in CML LSC, AKT activity is attenuated possibly explaining their quiescent phenotype. The observation that TGF-β1 is capable of modulating AKT activity and inducing quiescence in CML LSC would suggest that it might play a key role in both of these processes in CML LSC. In the Naka report this putative mechanism underlying TGF-β1 effects on CML LSC maintenance was taken further by showing that TGF-β1, by reducing AKT phosphorylation, caused reactivation of FOXO3a transcription factor which plays a central role in both HSC[120] and LSC[111] maintenance; however while this was not demonstrated in the experiments presented, it is worth noting that recent evidence from our lab shows that p-AKT levels are lower with a concurrent activation of FOXO3a transcription factor in human CML LSCs compared to LPCs[435]. This observation provides a possible link between p-AKT levels and FOXO activity in the primary setting too and renders it possible to speculate that a similar pathway to the one shown in mouse CML LSC is also active in primary CML LSC following TGF-β1 stimulation (figure 6-11).

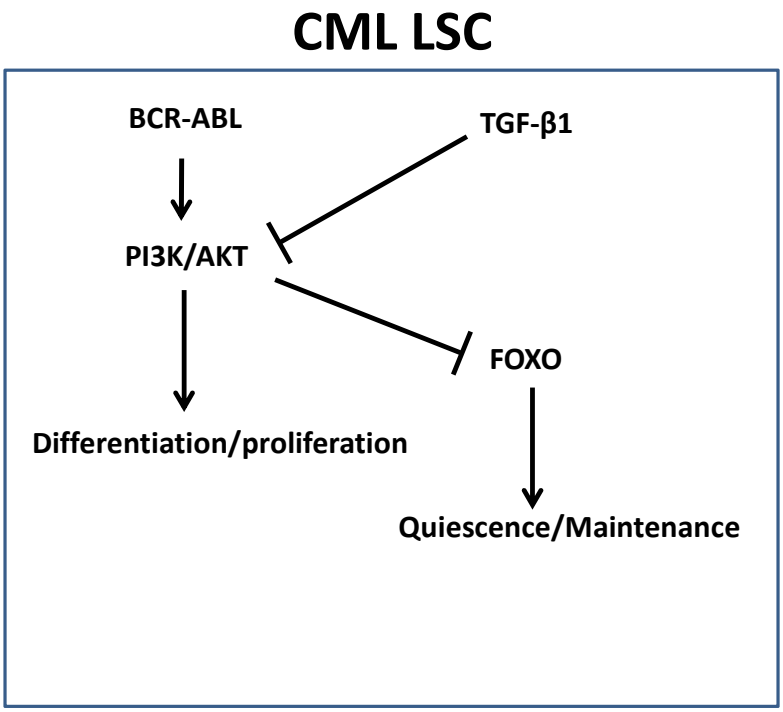


Figure 6-11 Putative mechanism for reduced AKT activity and TGF-β1 induced quiescence in CML LSC

TGF-β1 signalling has been involved in maintenance of other CSC, such as glioma CSC, thus suggesting that its effects on stem cell maintenance might be more generalised and relevant to different cell contexts[436]. TGF-β1's ability to also regulate normal HSC quiescence leads to the central question if its effects on inducing CML LSC quiescence can be exploited therapeutically without detrimental effects on normal HSC maintenance. An interesting finding from the work presented was that normal HSCs appeared to be less sensitive to TGF-β1 stimulation than CML LSCs as the induction of quiescence in normal HSCs was rather limited upon TGF-β1 exposure. Consistent with this, AKT activity in normal HSCs was already very low and did not decrease any further upon TGF-β1 exposure. This is consistent also with the observations from Naka *et al* where CML mice treated with TGF-β1 R1 kinase inhibitor alongside IM showed prolonged survival with no evidence of toxicity to normal HSCs. This suggests a preferential effect of TGF-β1 in controlling quiescence of CML LSC versus normal HSC, via its effects on AKT activity. Another report has also suggested that the presence of BCR-ABL upregulates TGF-β1 signalling pathway activity in CML cells and this could contribute to their increased sensitivity to TGF-β1 exposure[433]. Finally, it has long been known that while normal HSCs are responsive to a variety of negative cell cycle regulators, such as TGF-β1 and chemokines, such as MCP-1[259], CML cells are only responsive to some of the above regulators, such as TGF-β1. This would support a model where CML LSCs are more heavily reliant on TGF-β1 signalling relative to normal HSCs (possibly because of its

ability to control the constitutive AKT activity of CML LSC) as TGF-β1 is one of the few signalling pathways capable of controlling their quiescence given their lack of response to other negative regulators. Conversely in normal HSCs, their response to other negative cell cycle regulators can compensate for a lack of TGF-β1 signalling and preserve their quiescence. Taken together these observations suggest that inhibition of TGF-β1 signalling could preferentially push CML LSCs into cell cycle and make them sensitive to TKI treatment, while preserving normal HSCs quiescence, and could therefore be exploited therapeutically as supported by the results of Naka report in the CML mouse model (figure 6-12).

However, it has to be said that no evidence was provided in the experiments described for a therapeutic strategy targeting TGF-β1 signalling in CML and this is a limitation of the current experimental work which will require further investigation in the future, by combining TGF-β1 inhibitors with TKI, in both CML LSCs and normal HSCs, to assess their effects. In any case, application of TGF-β1 inhibitors to the treatment of CML warrants careful consideration mainly because a therapeutic strategy aiming to increase the proliferation of CML LSC carries the obvious risk of potentially leading to disease progression. It is worth noting that in their report Naka *et al* share, on similar grounds, the same concerns regarding such a therapeutic approach. Nevertheless it is indeed possible that in CML CP patients with minimal residual disease after long term TKI therapy, a therapeutic approach targeting TGF-β1 signals (or in that respect any other pathway central to CML LSC quiescence), together with TKI, might be safe and indeed capable of depleting CML LSCs leading to disease eradication and, as discussed in section 1.3.7.4.1, this is an approach currently being actively pursued in the CML research field which will continue to attract further investigation over the next few years.

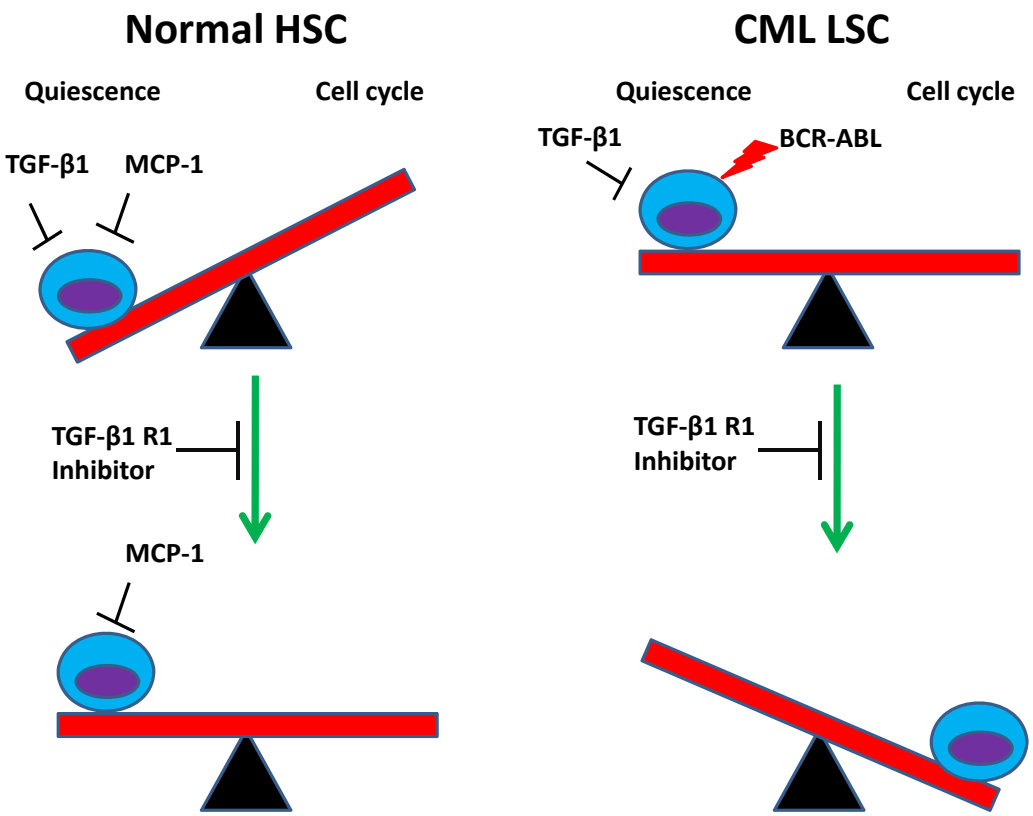


Figure 6-12 Putative model for the higher sensitivity of CML LSC to TGF-β1 signalling
Normal HSC proliferation is controlled through multiple factors (such as TGF-β1, MCP-1 and others). In CML LSC, the presence of BCR-ABL reduces their responsiveness to some of these negative regulators, such as MCP-1. As a result, upon inhibition of TGF-β1 signalling, CML LSC are more easily pushed into cycle compared to normal HSC which can still rely on the control provided by other active negative regulators. This could provide a therapeutic advantage as CML LSC could be preferentially pushed into cycle by TGF-β1 inhibition and exhausted with little or no detrimental effect to normal HSC maintenance.

7 Final conclusions and future directions

CML represents to date the most paradigmatic tumour in the field of cancer research. Since its original description, it was the first cancer for which a specific and consistent genetic lesion was demonstrated and subsequently shown to be pathogenic. Many other cancers are now being shown to have similarly specific and recurrent genetic abnormalities and with improved technology it is possible that such a feature will be recognised in the majority, if not almost all, known cancers enabling classification and diagnosis on more reliable genetic grounds rather than the currently used morphological features[371,437]. CML is also a paradigmatic example of a cancer following a stem cell model and as such represents an elegant disease model to study cancer stem cell biology. Finally CML is also the first disease where a better understanding of the tumour cell biology led to the development of rationally designed, targeted therapy and which again paved the way for similar developments in many other forms of both solid and haematological tumours[372,438]. These are probably the main reasons why CML, despite the fact that it is currently an area of less clinical need compared to other forms of cancer, still attracts the attention of so many researchers.

Recently one of the main focuses of research in the CML field has been trying to understand if CML LSCs are "oncogene addicted". This term, first coined by Weinstein, refers normally to the passive dependence of tumour cells on a particular activated oncogene for maintenance of their malignant phenotype and was originally illustrated using transgenic mouse models, whereupon inactivation of the oncogene, all tumour cells underwent proliferative arrest and/or apoptosis[439]. The situation in humans however might be more complex than in mouse models, partly because in mouse models oncogenes play an unusually potent role, and also because in humans the carcinogenic process is longer, thus allowing additional genetic/epigenetic changes to happen in cancer cells that might reduce their dependence on the oncogene. Nevertheless in CP CML, *BCR-ABL* represents the active oncogene in all patients and also a relevant therapeutic target given its pathogenic role which might support the notion that all CML cells are oncogene addicted. However, the clinical observations that, despite prolonged TKI therapy, CML stem cells persisted in patients' BM, coupled with the *in vitro* insensitivity of the same cells to all currently used TKI (fully reviewed in section 1.3.7.4), has led many investigators to question the actual relevance of the *BCR-ABL* oncogene in the specific survival of LSC.

Two recent papers have addressed this issue and, using different assays and complementary models, came to the same conclusion, i.e. CML LSCs are not *BCR-ABL* oncogene addicted, or more precisely addicted to its TK activity[337,338]. The immediate consequence of this finding is that BCR-ABL TK alone is no longer a legitimate target for the eradication of CML LSCs and novel therapeutic targets intrinsic to the CML LSCs have to be found to achieve disease cure. This has inevitably opened the research field in CML as now several novel avenues have to be investigated to understand specific LSC survival mechanisms. It is worth noting that this is also likely to have applications in other forms of cancer and will possibly be one of the reasons why CML will continue to be a paradigmatic disease in the future.

With the current development of high throughput technologies and the emergence of a systems biology approach to biological research, a current method used to direct research in CML LSC biology has been to obtain, analyse and integrate, often using mathematical modelling, complex data sets generated using various technology platforms from different experimental sources. This means integrating information from genome, epigenome, transcriptome and proteome-wide data sets to identify specific features within a biological system (in this case CML LSC) to generate experimentally testable hypotheses which can further the understanding of the functions of specific gene/protein/pathways within a cell. The great advantage of such an approach is its unbiased nature which can, for example, highlight otherwise unknown mechanisms necessary to the survival of a LSC and which can be exploited therapeutically.

The experimental work presented in this thesis fits into the wider perspective of understanding novel survival mechanisms of CML LSC. Moreover it stems from a combination of systems biology approach and more traditional hypothesis driven research. The interest in the role of GFs signalling in CML biology and pathogenesis is longstanding as it originates from original observations of the autocrine production of GFs in both BCR-ABL positive cell lines and primary $CD34^+$ cells[270], and from the observations that CML SPC, although normally responsive to some GF signals, also show aberrant responses to others, which might explain their highly proliferative behaviour[259]. More recently however, results of genome and epigenome-wide screening performed in our laboratory have also highlighted the potential role of various known and novel GFs [N.B. as already mentioned in the Introduction the term GF/cytokine are used here in an interchangeable fashion to include any extrinsic regulator of SPC] in CML LSC maintenance, thus confirming, using

an unbiased screen, the validity of an investigation of the role of GFs signalling in CML LSC biology, while also providing novel specific research areas to focus on.

Therefore the overall aim of this thesis was to investigate in further detail the role of known and novel GFs and their downstream pathways in CML SPC biology. The work started by characterising further the autocrine expression of GFs and cognate receptors by CML SPC relative to normal SPC, and in response to BCR-ABL kinase inhibition, to identify candidate GFs or downstream signalling pathways on which to concentrate the ensuing work (chapter 3). The JAK2/STAT5 pathway as the downstream common signalling pathway of several GFs produced in an autocrine fashion by the CML cells relative to normal (chapter 4), and the BCR-ABL kinase independent autocrine TNF-α production by CML SPC (chapter 5), were thus identified respectively as a candidate signalling pathway and GF to study further. In particular their function in survival, proliferation and maintenance of CML SPC was tested mainly in the context of BCR-ABL kinase inhibition to try and validate their role as legitimate and exploitable therapeutic targets which specifically contribute to CML SPC survival in a BCR-ABL kinase independent fashion. The responses to the inhibition or stimulation of the same candidate GFs and signalling pathways were also assessed in normal SPC to test if any difference could be detected in the sensitivity of normal SPC to the modulation of these GFs signalling pathways to predict the potential toxicity of any therapeutic approach targeting them. Finally, starting from the results of an unbiased screen, the putative role of novel neurotransmitter signalling pathways, specifically in CML LSC proliferation, viability and self-renewal, was also investigated (chapter 6).

A detailed discussion of the research findings for each studied GF or downstream pathway function in CML SPC survival, proliferation and maintenance was presented at the end of each result chapter. Therefore in this final discussion, some overall conclusions stemming from the whole of the work presented in this thesis will be discussed.

7.1 GF signals control several aspects of CML SPC biology and interact with each other: identification of common therapeutically exploitable signalling hubs

The overall work presented in this thesis supports a role for several GFs, whether produced in an autocrine fashion or by BM stromal cells, in supporting CML cell survival, proliferation and maintenance, even when the BCR-ABL kinase activity is inhibited. One overall interesting observation from the presented work is that there is evidence of crosstalk between different GFs signals with each of them potentially contributing to stimulate/inhibit through positive and negative feedback, the activity of the others. The ability, for example, of TNF-α to upregulate CSF2RB in CML $CD34^+$ while also upregulating the mRNA expression of several GFs, such as *GM-CSF* and *SCF*, supports such a model. This observation suggests the existence of a more complicated network of GF interactions which requires further investigation to be fully elucidated. In this regard, it is also worth noting that GF signalling pathways often directly overlap or interact with each other, and that in CML cells, a physical interaction via BCR-ABL between JAK2/AKT and NFκB leading to modulation of MYC expression has already been shown[366] that might be relevant in the crosstalk between different GFs previously mentioned. In any case a better understanding of these interactions could be potentially therapeutically exploitable if relevant key players amongst the GFs and downstream signals are identified which allow several potentially important pathways to be targeted at once.

JAK2 has emerged from the work presented as one such possible key player given not only its ability to relay signals from a variety of GFs, but also the fact that its inhibition appeared to modulate the expression of other GFs, such as TNF-α and the IL-4 receptor. Moreover JAK2 inhibition would also reduce signalling emanating from CSF2RB, which is upregulated by TNF-α itself. Although JAK2 is a possible example of a central signalling hub, further investigations could still help to identify both other relevant hubs within GFs signalling pathways, which should be targeted therapeutically, and potential resistance mechanisms of CML SPC to the therapies tested. Such investigations could benefit grossly from unbiased gene expression and (phospho)proteomics screens as shown only in a preliminary fashion by the presented JAK2 inhibitor + TKI proteomic screen which is currently being extended to the phosphoproteome.

In this regard, it is noteworthy that for example BCL6 and MYC have emerged as possible examples for, respectively, resistance mechanism and relevant downstream target in the work presented. BCL6, which recently was shown to be central to CML LSC resistance to TKI and have a central role in self-renewal signals of CML LSC[378], was upregulated following JAK2 inhibitor and TKI combined treatment, highlighting a potential resistance mechanism to this treatment approach by CML SPC whose relevance warrants further investigation. This observation suggests that, despite its efficacy, JAK2 inhibition combined with TKI might still encounter resistance in a proportion of patients. Conversely the observation that both, JAK2 inhibition plus TKI and TNF-α inhibition plus TKI led to downregulation of MYC expression is an interesting finding which highlights MYC as a relevant therapeutic target in CML $CD34^+$ cell survival. MYC has been reported to be central to BCR-ABL transforming capacity for a long time[247], however only recently has its importance in CML cells as a central survival hub been fully appreciated[366]. A recent report using unbiased combined proteomic and gene expression analysis has shown MYC, in BCR-ABL positive cell lines, to be the convergence point of combined treatment with the TKI BOS and the multikinase inhibitor danusertib, while either agent alone was ineffective[384]. Also, in that setting, combined treatment was particularly effective in eliminating BCR-ABL positive cells carrying a multiresistant T315I mutation.

Recently, MYC was shown to be a central transcription factor whose expression is under the control of epigenetic regulators often aberrantly regulated in AML cells. Newly developed targeted drugs interacting with these epigenetic regulators have now been shown to reduce MYC mRNA and protein expression and the activation of its downstream pathways necessary for maintenance of AML LSC, thus reinforcing the role of MYC in a closely related haemopoietic malignancy[417]. Its role in CML LSC survival needs further investigation. Indeed, recent work carried out in our laboratory in collaboration with Professor Tony Whetton in Manchester, using an extensive unbiased proteomic and phosphoproteomic screen, has shown that in CML LSC, several pathways under the control of MYC are more active relative to normal HSC, thus also suggesting in the primary CML model the importance of MYC to CML LSC survival. Although MYC transcription factor in itself has been always difficult to target because of the lack of a druggable enzymatic activity, the recent aforementioned development of novel drugs acting on the epigenetic regulation of its expression have made its therapeutic targeting possible. Initial results of work carried out in our lab using such drugs are indeed showing promise, as interference with MYC activity appears to specifically and effectively target CML SPC, while sparing normal counterparts (unpublished data).

7.2 Therapeutic exploitation of GF survival signals: advantages and pitfalls

Overall the work presented in this thesis suggests that therapeutic targeting of various GF signals, or of their downstream pathways, is potentially helpful in improving the eradication of CML $CD34^+$ cells when combined with standard TKI therapy. While this is potentially opening the opportunity to novel treatment options for CML patients (particularly as some of the drugs shown in this thesis to be effective *in vitro* have already reached clinical development), concern remains regarding the safety of all these treatment approaches. GF signals are often relevant to normal HSC survival and proliferation and indeed there is great overlap between CML and normal $CD34^+$ cells in their reliance on these signals for their survival. Potential toxicity to normal $CD34^+$ cells of some of the treatment approaches investigated in this thesis have been highlighted. These considerations stress the importance of improving our understanding of the differences in production and response to GFs of CML cells compared to normal.

Autocrine production is indeed a mechanism which appears more relevant in some cases to CML cells. For example, the high level autocrine TNF-α production is peculiar to CML cells and in this context is mainly relaying proliferative and survival signals, therefore providing a potentially specific and hence non toxic therapeutic target. Moreover, it might even be possible to exploit high autocrine TNF-α production by CML $CD34^+$ cells indirectly if its effects are redirected towards induction of apoptosis, by targeting the mechanisms which allow TNF-α to relay survival signals, such as NFκB activity and expression of IAPs, as shown in other cancer systems[401]. Another example is given by the higher expression of CSF2RB in CML compared to normal cells which would suggest that JAK2 signalling might indeed be more active in CML cells, thus providing a therapeutic window there too. These are just some of the examples originating from this work that show how a better understanding of the peculiarity of CML cell production and response to GFs might be exploited therapeutically. Therefore while it is likely that targeting some of these signalling pathways might inevitably lead to a certain degree of toxicity towards normal HSPC, it is possible that an improved understanding of the exact role of each GF in CML SPC biology relative to normal and its characterisation at the molecular level might render possible the identification of novel specific therapeutic targets. Further investigations are definitely warranted in this respect.

This issue is particularly relevant when mechanisms central to CML LSC maintenance and survival are identified, as it is likely that therapies targeting these mechanisms will be mainly employed in the eradication of persistent low level residual disease. It is therefore paramount to ensure that no damage to normal haemopoiesis occurs as a result of treatment approaches targeting CML LSC maintenance/survival pathways as this would potentially result in harm to a group of patients which is currently enjoying good responses and quality of life on TKI treatment. In this regard it is always important to compare directly in each case CML SPC and normal HSPC responses to the different treatment approaches investigated as was done most of the time in the work presented. Nevertheless, the final proof of lack of toxicity of any treatment approach will require *in vivo* data which obviously represents a limitation of the work presented. However when possible, the confirmation of any *in vitro* finding through *in vivo* data was sought through active collaboration with other groups active in the research field and in the future, as our laboratory animal facility develops, this will become possible in each case. In conclusion, although therapeutic exploitation of targeting GFs signals was shown in this thesis to be possible, particularly for those GFs differentially produced by CML cells, or for which the CML cell showed higher sensitivity, further confirmation of these findings is awaited through *in vivo* experiments currently underway or planned for the future and therefore a degree of caution is required before supporting these as possible treatment approaches.

7.3 Novel potential regulators of CML LSC quiescence/maintenance: avenues for future work

CML LSC quiescence/self-renewal mechanisms, which have been shown to be BCR-ABL kinase independent, are amongst the main reasons behind CML LSC persistence in patients harbouring minimal residual disease on long term TKI therapy. In some of the work presented in this thesis the potential role of TGF-β1 and novel neurotransmitters in the control of both quiescence and self-renewal has been investigated. An interesting observation, which was common to both the neuromediator and TGF-β1 work, was the modulation of *CDKN1A/p21*, which suggests that this regulator of cell cycle can indeed play a central role in CML LSC quiescence/maintenance. *Cdkn1a/p21* is known to control normal HSC maintenance, particularly under stressful conditions[95]. More recently however its role in AML LSC, and possibly colon cancer initiating cell maintenance, has been shown and it appears to be secondary to its ability to reduce DNA damage normally caused by the expression of oncogenes in malignant cells[383,440]. Its potential role in CML LSC maintenance is, however, novel. Given its recurrent appearance amongst the mechanisms of action of several putative regulators of CML LSC quiescence and self-renewal, its role could be central in this process and is worth further investigation in the future. Together with *CDKN1A/p21*, *ID1* (an inhibitor of basic helix-loop-helix transcription factors) gene expression levels also appeared to be upregulated following NEPI and ACH treatment. Moreover its levels were downregulated by combined JAK2 inhibitor therapy and TKI and correlated with toxic effects on CML SPC. These results taken together suggest a potential role of *ID1* in CML SPC survival and potentially also maintenance of more primitive LSC.

ID1 has been shown to play a central role in HSC self-renewal [380,381] with more recent reports also supporting its potential role in promoting a myeloproliferative disorder *in vivo*[382] and in maintaining colon cancer initiating cells[383]. Interestingly in the latter report, ID1 effects appeared to be secondary to its ability to upregulate CDKN1A/p21 expression, thus providing a speculative link between these two proteins. The simultaneous upregulation of both ID1 and CDKN1A/p21 by NEPI and ACH treatment raises the question of whether a similar mechanism to the one present in colon cancer initiating cells is also present in CML LSC. Its role in CML LSC therefore warrants further investigations, including its potential link to CDKN1A/p21 function. In a similar manner to the importance of identifying central hubs common to different GFs pathways (such as MYC) when trying to therapeutically exploit GFs survival signalling, the identification of novel key regulators of CML LSC quiescence and self-renewal is necessary when trying to

understand the mechanisms of these processes. Both ID1 and CDKN1A/p21 appear to be two biologically plausible candidate regulators of these processes based on the results presented and published literature and might attract further future investigation as central convergence points of different signals regulating CML LSC quiescence and self-renewal.

In conclusion, since its original description almost two centuries ago by Bennett[441] and Virchow[442], the CML research field has witnessed unparalleled progress in the understanding of the pathogenesis and, as a result, in the management of this condition. However the ultimate goal of disease cure achieved through non toxic and targeted therapies has still eluded the CML community. The recent clarification that CML LSCs are not BCR-ABL kinase dependent for their survival has however generated a revived effort to improve our understanding of the biology of LSC in the hope that this will lead to the development of therapies targeted towards this highly resistant (at least to current treatment approaches) population. It is now believed in the CML research community that only an approach combining a TKI to reduce disease burden and an agent to target the LSC is likely to achieve CML cure. The work presented in this thesis fits in this bigger perspective by highlighting the contribution of GFs to the survival, proliferation and maintenance of CML SPC, while also investigating possible ways of therapeutically targeting some of these candidate GF signals. It is likely that at least some of these novel therapeutic strategies will reach clinical testing in the near future. Meanwhile more bench work, partly originating from some observations presented in this thesis, will be carried out in the future to clarify in further detail the role and interaction of these and novel GFs in CML SPC survival. This will help to improve our ability to target GFs survival signals in a more specific and effective fashion to eradicate the CML SPC.

Appendix I

Custom designed PCR primer sequences

GENE	FORWARD PRIMER	REVERSE PRIMER	ANNEALING T°/ NUMBER OF CYCLES	EXPECTED AMPLICON SIZE
GM-CSF	AACCTGAGTAGA GACACTGC	GATAATCTGGG TTGCACAGG	60°/40	225 basepairs (bp)
HGF	GAGTGGCATCAA ATGTCAGC	TCGATAACTCT CCCCATTGC	60°/40	214/229 bp (2 splicing variant)
IL-2	CAAACCTCTGGA GGAAGTGC	ATGGTTGCTGT CTCATCAGC	60°/40	153 bp
IL-4	GTGCGATATCAC CTTACAGG	CACAGGACAGG AATTCAAGC	58°/40	298/250 bp (2 splicing variant)
SCF	TCATTCAAGAGC CCAGAACC	TAAGGCTCCAA AAGCAAAGC	58° /40	330/240 bp (2 splicing variant)
TGF-β1	ACTGCAAGTGGA CATCAACG	TGCGGAAGTCA ATGTACAGC	60°/40	218 bp
TNF-α	CCTCAGCCTCTT CTCCTTCC	GGCTACAGGCT TGTCACTCG	60°/40	148 bp

TaqMan Gene expression assays

GENE NAME	ASSAY ID
ADRA2A	Hs01099503_s1
APO-E	Hs00171168_m1
BCL6	Hs00277037_m1
BCL-X_L	Hs01067345_g1
CDKN1A/p21	Hs00355782_m1
CDKN1B/p27	Hs00153277_m1
CHRM3	Hs00327458_m1
CSF2RB	Hs00166144_m1
Cyclin D1	Hs00765553_m1
Cyclin D2	Hs00277041_m1
Cyclin D3	Hs00426901_m1
GAPDH	Hs99999905_m1
G_i	Hs01053355_m1
GM-CSF	Hs00929873_m1
GUSB	Hs99999908_m1
HGF	Hs00300159_m1
HPRT1	Hs02800695_m1
IAP1	Hs01112284_m1
IAP2	Hs01112284_m1
ID1	Hs03676575_s1
IL-4	Hs00174122_m1
IL-4Rα	Hs00166237_m1
MCL1	Hs03043899_m1
MYC	Hs00905030_m1
PLCB1	Hs00248563_m1
PRKCE	Hs00178435_m1
PTEN	Hs03673482_s1
SCF	Hs01030222_m1
SURVIVIN	Hs04194392_s1
TBP	Hs99999910_m1
TGF-β1	Hs00998129_m1
TGF-β1R1	Hs00610320_m1
TGF-β1R2	Hs00234253_m1
TNF-α	Hs99999043_m1
XIAP	Hs01597783_m1

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